Mechanisms of disease

Effect of a bacterial pheromone peptide on host chemokine degradation in group A streptococcal necrotising soft-tissue infections

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Summary

Background Necrotising soft-tissue infections due to group A streptococcus (GAS) are rare (about 0·2 cases per 100 000 people). The disease progresses rapidly, causing severe necrosis and hydrolysis of soft tissues. Histopathological analysis of necrotic tissue debrided from two patients (one with necrotising fasciitis and one with myonecrosis) showed large quantities of bacteria but no infiltrating neutrophils. We aimed to investigate whether the poor neutrophil chemotaxis was linked with the ability of group A streptococcus (GAS) to degrade host chemokines.

Methods We did RT-PCR, ELISA, and dot-blot assays to establish whether GAS induces synthesis of interleukin 8 mRNA, but subsequently degrades the released chemokine protein. Class-specific protease inhibitors were used to characterise the protease that degraded the chemokine. We used a mouse model of human soft-tissue infections to investigate the pathogenic relevance of GAS chemokine degradation, and to test the therapeutic effect of a GAS pheromone peptide (SIiCR) that downregulates activity of chemokine protease.

Findings The only isolates from the necrotic tissue were two β-haemolytic GAS strains of an M14 serotype. A trypsin-like protease released by these strains degraded human interleukin 8 and its mouse homologue MIP2. When inoculated subcutaneously in mice, these strains produced a fatal necrotic soft-tissue infection that had reduced neutrophil recruitment to the site of infection. The M14 GAS strains have a missense mutation in the start codon of silCR, which encodes a predicted 17 aminoacid pheromone peptide, SIiCR. Growth of the M14 strain in the presence of SIiCR abrogated chemokine proteolysis. When SIiCR was injected together with the bacteria, abundant neutrophils were recruited to the site of infection, bacteria were cleared without systemic spread, and the mice survived. The therapeutic effect of SIiCR was also obtained in mice challenged with M1 and M3 GAS strains, a leading cause of invasive infections.

Interpretation The unusual reduction in neutrophils in necrotic tissue of people with GAS soft-tissue infections is partly caused by a GAS protease that degrades interleukin 8. In mice, degradation can be controlled by administration of SIiCR, which downregulates GAS chemokine protease activity. This downregulation increases neutrophil migration to the site of infection, preventing bacterial spread and development of a fulminant lethal systemic infection.

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Introduction

Group A streptococcus (GAS) is a gram-positive pathogen that often causes mild non-invasive throat and skin infections and life-threatening highly invasive diseases, including toxic-shock syndrome and necrotising soft-tissue infections.1 Necrotising soft-tissue infection consists of a range of rapidly progressive diseases including necrotising fasciitis, myonecrosis, and acute streptococcal rhabdomyolysis. Historical accounts of necrotising soft-tissue infections abound, including Claude Pouteau’s description in 1783 and Melney’s detailed analysis of acute haemolytic streptococcal gangrene in 1924.2

Published series from several countries have cited an increase in reports of toxic-shock syndrome and necrotising fasciitis due to GAS infections in the past two decades.1 However, this increase might be the result of better recognition of the disorders by doctors or improved population-based surveillance, rather than a true change in epidemiology.3

Despite prompt antibiotic treatments and surgical debridement, GAS toxic-shock syndrome and necrotising fasciitis are associated with high death rates ranging from 20% to 60%.4 Renewed appreciation of highly invasive GAS infections has rekindled interest in the basic mechanisms of GAS pathogenesis and spurred efforts to identify and develop new treatment approaches.1

Necrotising GAS soft-tissue infections can be produced by various serotype strains,1 although M1 and M3 have been the most prevalent in the larger published series from the UK,5 Canada,6 and the USA.7 In a prospective, population-based study of invasive GAS infections in Israel,8 we found a high proportion of M14 strains in those causing severe soft-tissue infections. The M14 strains could be isolated from cases with pneumonia, necrotising fasciitis, skin abscess, and from throat cultures taken from close contacts of index cases.9 M14 type strains were also isolated from healthy schoolchildren.10

The emergence of very widespread and virulent GAS clones suggested that the increased invasiveness might be associated with specific genetic elements that have been acquired by horizontal transmission11 or phage acquisition.12 Conversely, that genetically indistinguishable clones can be isolated from invasive and non-invasive infections suggests that host factors might play a part in determining disease outcome.13 Korb and colleagues14 suggested that allelic variation of HLA II contributes to the differences in severity of GAS infections.
Analysis of an M14 GAS strain isolated from a patient with necrotising fasciitis led us to identify and characterise the streptococcal invasion locus, \textit{sil}, which is highly homologous to a regulon of \textit{Streptococcus pneumoniae} involved in bacterial signalling. Within this locus is a gene, \textit{silCR}, predicted to encode a 41-aminoacid propeptide. \textit{SilCR} has characteristics of a bacterial \textit{pheromone peptide}, including a conserved cleavage site predicted to generate a 17-aminoacid mature peptide\textsuperscript{15} (webfigure 1, http://www.thelancet.com/extras/03art9330webfigure1.pdf). However, in M14 GAS, a \textit{MISSENSE MUTATION} changes the ATG start codon of \textit{silCR}, suggesting that the peptide might not be produced.\textsuperscript{15} \textit{SilCR} with an ATG start codon is present in the genome of M18 GAS,\textsuperscript{17} but one of the predicted transporter genes of the pheromone \textit{silD} is truncated.\textsuperscript{15} \textit{SilCR} is absent in the M1 and M3 GAS genomes. We speculated that \textit{SilCR} might have a role in regulating GAS's ability to cause invasive infection.

Here we report findings for two patients with necrotising soft-tissue infections caused by M14 serotype GAS strains.

**Methods**

**Patients**

A 40-year-old man who was previously healthy was admitted 1 week after sustaining a minor injury to the left elbow, with fever, excruciating pain, and swelling of the arm. CT examination of the arm showed infiltration of the subcutaneous fat around the elbow consistent with cellulitis and fasciitis. We gave the patient intravenous cefazolin. Over the next few hours his general condition deteriorated and his blood pressure dropped to 70/40 mm Hg. We made a surgical incision from the mid forearm to the mid upper arm over the ulnar aspect. The fascia was necrotic with a noticeable sparing of underlying muscle. We did a fasciotomy (figure 1). The wound was left open with wet saline dressings. GAS was the only organism isolated from fluid aspirated from the elbow before surgery, from blood culture, and from tissue obtained during surgery. Histopathological analysis showed necrosis of fascia, and abundant bacteria but no neutrophil infiltration (figure 2). Treatment was changed to penicillin and clindamycin. The patient's blood glucose concentrations during his severe infection were occasionally slightly higher than normal with no urinary glucose. The patient was supported in the intensive care unit for 6 days and he gradually recovered. Over the following 10 days he had a repeated debridement of the wound. Cultures taken from tissue did not grow GAS. Once granulation tissue covered the wound, we applied a skin graft (figure 1), and the arm regained complete function. At follow-up his blood glucose concentration was normal.

The second case was an 84-year-old woman with diabetes mellitus on chronic steroid therapy for polymyalgia rheumatica. She was admitted because of...
fever and mental status changes for 3 days. On examination she was lethargic, tachycardic, with normal blood pressure. Her right foot had chronic dry necrotic ulceration of two toes. The calf was swollen with several haemorrhagic bullae. Her leucocyte count was 2·2×10^9/L with normal platelets. Clindamycin and ciprofloxacin were started. Blood cultures taken on admission grew GAS. Over the next 3 days the patient’s leg became progressively more swollen with signs of necrosis. On her fifth hospital day we amputated her leg above the knee. Histopathological analysis showed widespread regions of necrotic muscle and subcutaneous tissue with many thrombosed blood vessels. In the region of muscle necrosis an abundance of bacteria was noted without a neutrophil infiltration. A culture taken from deep tissue of the amputated leg was negative. After surgery the patient developed pneumonia and despite treatment in the intensive care unit died 2 weeks after admission.

**Laboratory analysis**

The Hadassah Hospital clinical microbiology laboratory identified the isolates from the surgically debrided tissues and blood as GAS by standard procedures. The M14 identification was made by serological typing at the Israel Ministry of Health Streptococcal Reference Laboratory and verified by M-protein genotyping. The presence of the sil locus in these strains was confirmed by PCR as previously described, and the characteristic missense mutation changing the silCR start codon was verified by direct DNA sequencing of the PCR amplicon. The GAS isolate from the first patient (JS95) was selected for further studies. Typically, GAS strains were cultured in Todd-Hewitt broth supplemented with 0·2% (weight/weight) of yeast extract, THY. Because the THY medium itself degrades chemokines, the assays of chemokine proteolysis were done in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. To eliminate residual chemokine proteolysis by fetal calf serum, the serum was pretreated with phenylmethylsulfonyl fluoride (PMSF) followed by fetal calf serum. To eliminate residual amounts of THY, which could cause proteolysis, THY was starting and eliminated by adding this amount of THY to the DMEM containing 10% fetal calf serum to eliminate proteolysis. Both fractions were then incubated until they reached an optical density at 600 nm of 0·3. Bacteria were washed twice with sterile phosphate-buffered saline and once with DMEM containing 10% fetal calf serum to eliminate residual amounts of THY, which could cause proteolysis. The washed pellets were resuspended in DMEM supplemented with fetal calf serum to their original volumes. We added 50 mg/L SilCR to the fraction that was to be grown in the presence of SilCR. The two fractions were then incubated for 1 h at 37°C. Supernatants were obtained after centrifugation at 42 000 g for 20 min. The proteolysis reaction contained 0·08 mL of each supernatant, 100 ng recombinant human interleukin 8/CXCL8 (R&D systems, Minneapolis, Minnesota) or 30 ng recombinant mouse MIP2 (R&D systems), 20 mmol/L 3-(N-morpholino)propanesulfonic acid (MOPS) pH 7·2 in a final volume of 0·1 mL. The reaction was done for 1 h at 37°C, and stopped by boiling for 1 min. For testing protease inhibitors, we preincubated the supernatants with the inhibitor for 30 min at 37°C, then did the proteolysis reaction. We used 4 mmol/L Pefabloc SC, 0·5 mmol/L PMSE, 0·3 μmol/L aprotinin, 1 mmol/L benzamidine, and 100 mg/L soybean trypsin inhibitor as inhibitors. We measured interleukin 8 content by ELISA using the Quantikine kit (R&D Systems), in accordance with the manufacturer’s instructions. Interleukin 8 is a potent C-X-C family chemokine and the best studied neutrophil chemoattractant in human beings. Since we have shown that group B streptococcus stimulates transcription and release of interleukin 8 from human A549 lung epithelial cells, we used this assay to measure the ability of JS95 of the invasive GAS M1-serotype strain 5448 to stimulate transcription and secretion of interleukin 8.

For dot-blotting, we diluted samples of 50 μL from the proteolysis reaction in phosphate-buffered saline to a final volume of 0·5 mL and passed them through a 96-well dot-blotter onto a 0·2-μm-nitrocellulose membrane. We detected MIP2, a functional homologue of human interleukin 8, with polyclonal antimouse MIP2 (R&D Systems) followed by addition of antimouse IgG-horseradish peroxidase. Dots were detected with the SuperSignal West Pico chemiluminescent detection kit (Pierce, Rockford, IL, USA).

**Role of the funding source**

The sponsors had no role in study design, data collection, data analysis, data interpretation, writing of the report, or the decision to submit the paper for publication.

**Results**

Tissues obtained by surgical debridement from patient 1 with necrotising fasciitis and patient 2 with myonecrosis were characterised by large amounts of bacteria and no infiltrating neutrophils (figure 2). By contrast, the viable tissue surrounding the site of necrosis contained neutrophils but no bacteria. Mice injected with 10⁶ cfu of M14 GAS strain JS95 became severely sick within 12 h, and after 24 h seemed lethargic, with mottled hair.
suggesting that interleukin 8 might be degraded by GAS. We tested whether a GAS-encoded proteolytic activity might be responsible for interleukin 8 degradation by incubating a supernatant of JS95 grown to early log phase with interleukin 8 in the absence and presence of class-specific protease inhibitors. Although JS95 supernatant effectively degraded interleukin 8, the irreversible serine protease inhibitor pefabloc SC almost abolished interleukin 8 degradation (webfigure 3, http://www.thelancet.com/extras/03art9330webfigure3.pdf). Aprotinin, which effectively inhibits trypsin, chymotrypsin, plasmin, and kallikrein, completely inhibited degradation of interleukin 8, suggesting that the protease responsible for such degradation is of the serine class (figure 6). Finally, benzamidine and soybean trypsin inhibitor also completely abrogated interleukin 8 degradation (data not shown).

When we grew JS95 in the presence of SilCR, the strain had greatly reduced interleukin 8 proteolytic activity suggesting that interleukin 8 might be degraded by GAS.

In mice sacrificed at 3 h after injection many bacteria appeared in the fascia which became necrotic without concomitant necrosis of the skin or subcutaneous tissues. We detected only a few neutrophils at the injection site, similar to control injections with phosphate-buffered saline alone. 6 h after challenge, necrosis extended from the fascia to the hair follicles. 12 h after challenge, extensive necrosis of fascia, dermis, and epidermis was observed, along with massive numbers of bacteria (webfigure 2, http://www.thelancet.com/extras/03art9330webfigure2.pdf). A paucity of neutrophil infiltration to the fascia and surrounding tissues was noted 12 h after inoculation, whereas neutrophils were absent 24 h after inoculation (figure 4, upper). These histopathological findings in people and mice suggested that the high invasiveness of the M14 GAS strains might in part reflect an impaired host neutrophil response that fails to contain the localised bacterial infection.

The JS95 and the invasive GAS M1-serotype strain 5448 both showed increased transcription of interleukin 8 mRNA in a dose-dependent manner, inducing a 3–4-fold increase over the baseline level at a bacterial load of 10^6 cfu (figure 5). However, despite the increased amount of interleukin 8 mRNA we did not find interleukin 8 in the culture medium of the A549 cells (figure 5),

Figure 3: Lesion in mouse after challenge with JS95 (upper) and with JS95 and SilCR (lower)
JS95 dose=10^8 colony forming units. SilCR dose=50 µg. Photo taken 24 h after challenge.

Figure 4: Haematoxylin and eosin stain of soft-tissue lesions from a mouse challenged with JS95 (upper) and with JS95 in presence of SilCR (lower)
JS95 dose=10^8 cfu. SilCR dose=50 µg. Magnification ×400. (Upper) Fascia shows extensive necrosis, with large presence of bacteria (blue), but no infiltration of neutrophils. Histopathological findings did not differ between four experiments each done in eight mice. (Lower) Fascia is inflamed and necrotic and contains many bacteria and neutrophils. Few neutrophils are present in muscle. Histopathological findings did not differ between four experiments in eight mice each.
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To retard neutrophil influx, GAS also produces an extracellular C5a peptidase, which cleaves the human neutrophil chemoattractant C5a. However, we did not see any change in the amount of surface expressed C5a peptidase in the absence and presence of SilCR (data not shown). GAS also produces the broad-spectrum cysteine protease SpeB, which plays a complicated part in pathogenesis through degradation of host tissue proteins, while autodegrading bacterial virulence factors such as M protein and secreted superantigens. We found that an isogenic SpeB-negative mutant of GAS M1 strain 5448 degraded interleukin 8 equally to the parent strain (not shown). These results suggest that neither the GAS C5a peptidase nor the cysteine protease SpeB is responsible for degradation of interleukin 8.

We found infarcted blood vessels in tissue obtained from patients 1 and 2, through which the bacteria might start an invasive systemic infection. After reaching the circulation through the infarcted endothelium, GAS-like S. aureus are phagocytosed and some survive within neutrophils. Survival in neutrophils perhaps could facilitate GAS spreading to deeper tissue and organs. Subsequent spreading of GAS in necrotising soft-tissue infections causes thrombosis of vessels, resulting in gangrene. In the baboon model of GAS necrotising fasciitis, surviving baboons had an intense neutrophil influx into the site of inoculation, whereas those that died had no influx of neutrophils, and intravascular coagulation. Indeed, GAS has been shown to cause abnormalities of the intrinsic pathway of coagulation through activation of the contact system.

The therapeutic effect of SilCR is exerted through its interaction with the bacterium, since SilCR did not stimulate neutrophil recruitment itself nor did it protect mice when administered simultaneously at a site distant to the location of bacterial challenge. In mice infected with M1 and M3 strains causing human soft-tissue infections, we found that SilCR decreases the virulence of M1 and M3 strains, suggesting that the pheromone recognises a common sensor system of GAS, and activates an unidentified signalling circuit. The M1 and M3 strains do not have the complete sil locus. Also, initial analysis shows that the sil locus is absent from most GAS serotype strains. In addition, in the M14 strain, silCR is mutated, whereas in the M18 strain the transporter, SilD, is truncated. Furthermore, the entire sil locus might be situated on a pathogenicity mobile island, which had been lost from the M1 strains genome during the M1 evolution. Thus, we postulate that in many GAS strains sil has become degenerate during bacterial coevolution with the human host either by complete deletion of the sil island or by introduction of mutations that render its components non-functional. The absence of an intact sil locus does not necessarily predestine GAS strains to high invasiveness since GAS virulence is a complex multifactorial process. Several overlapping regulatory networks that are recognised might control interleukin 8 protease expression. A homology/signature sequence search against the five available genomes of GAS identifies three serine-proteases as putative candidates for the interleukin 8 protease: X-propyl-dipeptidyl aminopeptidase (PepXP), DcpB, and PrtS/CapA.

Our results show that SilCR acts as a GAS signalling molecule that modulates the pathogen’s virulence potential. The effect of SilCR is manifested at a crucial point of GAS interaction with the host innate immune response, namely phagocytosis and killing by neutrophils.
MECHANISMS OF DISEASE

RELEVANCE OF THIS PAPER TO PRACTICE

BACKGROUND

Many host and microbial factors combine to determine the outcome of bacterial infections. One possible mechanism contributing to invasive infection is bacterial interference with an effective host response.

This paper describes two patients with very severe soft tissue infections, one of whom died. In both patients, virtually no neutrophils were present at the site of infection despite extensive bacterial infiltration. This finding was replicated in mice, and was linked to the ability of the bacteria—a strain of Group A Streptococcus—to degrade a molecule (IL-8) produced by the host to attract neutrophils to infected tissues. These very virulent streptococci were found to have a defect in a gene (silCR) encoding a small signalling peptide. When intact, the SilCR peptide has a regulatory function that inhibits bacterial IL-8 degradation. Giving SilCR to mice infected with the same invasive streptococci as in the affected patients, restored neutrophil migration and allowed the mice to clear the infection.

IMPLICATIONS

As well as clarifying why patients with this type of bacterial infection become so ill, this discovery suggests that treating individuals affected with infections with the missing regulatory peptide may limit tissue injury produced by the invasive strains.

Our results also show the therapeutic potential of a bacterial pheromone peptide in treatment of an infectious disease. Deciphering of the mechanisms of action of SilCR might shed light on GAS pathogenesis and offer new therapeutic options for invasive GAS infections such as necrotising fasciitis and myonecrosis.

Contributors

A E Moses and A Peyser treated the patients. A Peyser did the surgery. C Hidalgo-Grass, A E Moses, and E Hanski designed and did some of the experiments. M Dan-Goor, J Jaffe, M Ravins, Y Eran, L Kwinn, and V Nizet did some of the experiments. A Peyser did the surgery. A Peyser did the surgery. A Peyser did the surgery. A Peyser did the surgery.

Conflict of interest statement

None declared.

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References


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Clinical picture

Gastric perforation in a newborn

F Pulzer, J Bennek, E Robel-Tillig, M Knüpfer, C Vogtmann

A preterm (27·3 weeks’ gestation and 1155 g) boy presented on day 6, 8 h after a nasogastric tube was inserted. He had progressive deterioration, tachycardia, signs of disturbed circulation, and a huge abdominal distension. Besides mechanical ventilation he had received infusion therapy, catecholamines, antibiotics, and enteral feeding. The straight position and localisation of the end of the nasogastric tube on the anteroposterior abdominal radiograph strongly supported a diagnosis of gastric perforation (figure A, arrows). A pneumoperitoneum was confirmed on the lateral view (figure B). Urgent laparotomy revealed the presence of a localised gastric leak, which was managed operatively with complete resolution on follow-up.

Traumatic alimentary tract perforations in children secondary to instrumentation, though rare, can occur at any age, especially in neonates and young infants, and the clinical symptoms can mimic necrotising enterocolitis. Awareness of such a possibility is essential for prompt management to be initiated.

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Predicted mature form of SilCR--DIFKLVIDHISMARKKK

Figure 1: SilCR belong to a competence-stimulating family of peptides (CSPs). Sequence alignment (aa) of representative members of CSPs family from different streptococcal species. The accession numbers of the corresponding CSPs are: Q54712, CAA0435, AAK76284, CAA04364, O33666, O33668, O33675, respectively. The aa sequence of SilCR, was deduced according to the genome sequence (RefSeq, NC_003485) of the M18 MGAS8232 strain. The letters with gray background represent identical aa.

Figure 2: Histopathology of soft-tissue derived from mice at different time points after challenge with JS95 strain. Haematoxylin and eosin stain of the soft-tissue lesions biopsy from a mouse challenged with $10^8$ CFU of JS95: 3 (A), 6 (B), and 12 (C) h after inoculation, respectively (200x)
Figure 3: **The SilCR peptide does not directly affect IL-8 degradation.** JS95 was grown in the absence and presence of SilCR (50 µg/ml). IL-8 was incubated with the indicated supernatant fractions and then subjected to 18% SDS-PAGE. The separated protein bands were electro-transferred to a nitrocellulose membrane. Anti-IL-8 antibody and a secondary antibody conjugated to horseradish peroxidase were used to detect IL-8. The number of the lane represents incubation of IL-8 with: 1, DMEM + FCS (control); 2, JS95 supernatant; 3, JS95 supernatant grown in the presence of SilCR; 4, JS95 supernatant, pretreated with pefabloc SC; 5, supernatant of JS95 grown in the absence of SilCR, then 50 µg/ml of SilCR was added to the degradation assay.

![Image of Figure 3](image-url)

Figure 4: **The protective effect of SilCR is dose-dependent.** Survival of mice inoculated subcutaneously with either 10⁸ cfu of JS95 (●) or with 10⁸ cfu of JS95 mixed with increasing amounts (µg) of SilCR: 3.1 (■), 6.2 (▲), 12.5 (●), 25 (■), and 50 (●). Mice were observed daily post inoculation. The experiments were repeated 3 times yielding similar results.

![Image of Figure 4](image-url)