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Rapid *in situ* chondrocyte death induced by *Staphylococcus aureus* toxins in a bovine cartilage explant model of septic arthritis

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SUMMARY

Objective: To assess *in situ* chondrocyte viability following exposure to a laboratory strain and clinical isolates of *Staphylococcus aureus*.

Methods: Bovine cartilage explants were cultured in the presence of *S. aureus* 8325-4 (laboratory strain), clinical *S. aureus* isolates or non-infected culture medium of pH values 7.4, 6.4 and 5.4. All clinical isolates were isolated from the joint aspirates of patients presenting with *S. aureus*-induced septic arthritis (SA). At designated time points, *in situ* chondrocyte viability was assessed within defined regions-of-interest in the axial and coronal plane following live- and dead-cell image acquisition using the fluorescent probes 5-chloromethylfluorescein diacetate (CMFDA) and propidium iodide (PI), respectively, and confocal laser-scanning microscopy (CLSM). Cartilage water content, following *S. aureus* 8325-4 exposure, was obtained by measuring cartilage wet and dry weights.

Results: *S. aureus* 8325-4 and clinical *S. aureus* isolates rapidly reduced *in situ* chondrocyte viability (>45% chondrocyte death at 40 h). The increased acidity, observed during bacterial culture, had a minimal effect on chondrocyte viability. Chondrocyte death commenced within the superficial zone (SZ) and rapidly progressed to the deep zone (DZ). Simultaneous exposure of SZ and DZ chondrocytes to *S. aureus* 8325-4 toxins found SZ chondrocytes to be more susceptible to the toxins than DZ chondrocytes. Cartilage water content was not significantly altered compared to non-infected controls.

Conclusions: Toxins released by *S. aureus* have a rapid and fatal action on *in situ* chondrocytes in this experimental model of SA. These data advocate the prompt and thorough removal of bacteria and their toxins during the treatment of SA.

Introduction

Septic arthritis (SA), defined as joint inflammation secondary to bacterial infection[1], can be a highly destructive disease[2]. In developed countries, the incidence of SA ranges from 2 to 10 cases/100,000 persons[3–6] and affects all age groups, with incidence peaks in children and the elderly[7]. Although any joint can be affected, there is a propensity for lower limb involvement[8,9]. The majority of cases arise through the haematogenous spread of bacteria to the joint[10] but direct joint-colonisation may also occur following intra-articular injections, surgical instrumentation or penetrating trauma[4].

Despite current treatment strategies, which include intravenous antibiotics coupled with joint lavage[5], retrospective studies indicate that some degree of permanent joint damage, involving direct cartilage injury, develops in up to 50% of cases[11–13]. This suggests that chondrocyte death may occur during SA, as these cells are the only living agents capable of cartilage maintenance[14]. However, chondrocyte loss and subsequent cartilage degeneration may not be clinically evident for years. Thus, it is possible that supposedly-treated SA may have seeded the foundations for future problems e.g., early-onset osteoarthritis.

Numerous bacterial species are capable of inducing SA[11]. The most commonly isolated organism however is *Staphylococcus aureus*, accounting for 40–65% of cases[15,16]. *S. aureus* is a highly virulent pathogen associated with a wide range of serious infections[17–19] and

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produces a diverse array of toxins[20]. Studies investigating a variety of *S. aureus*-related infections have implicated a group of exotoxins (including alpha(α)-, beta(β)-, gamma(γ)- and delta(δ)-haemolysin) as key virulence factors[17,18,21,22]. Murine models suggest that alpha- and gamma-haemolysin play an important role in the joint destruction associated with SA[19,23].

This study primarily focuses on *S. aureus* 8325-4, which is a laboratory 'wild-type' strain. It is a well-characterised prophage-cured derivative of NCTC8325[23,24] and produces the major *S. aureus* toxins[19]. NCTC8325 was originally isolated from a sepsis patient in 1960 and its lineage remains a valuable resource for *S. aureus* research[25]. In addition, we have investigated clinical isolates of *S. aureus*, isolated from the joint aspirates of patients presenting with *S. aureus*-induced SA.

Previous work investigating bacteria and associated toxins has primarily focused on isolated chondrocytes, cartilage explants or animal models[26,27]. Isolated chondrocytes have the advantage of allowing easier control of experimental conditions but have the disadvantage of not allowing the study of chondrocytes in their native extracellular environment. In contrast, chondrocytes within cartilage remain within their native environment but studies to date have utilised assays for either the release of extracellular matrix (ECM) components[27,28] or degradative enzymes[29] as experimental end-points. Although these provide an important indication of cartilage damage, they give no information on chondrocyte viability following bacterial exposure. An *in vitro* model of SA whereby *in situ* chondrocyte death can be both directly visualised and quantified, whilst at the same time allowing better control of experimental variables, would be highly desirable.

It is likely that there are two major mechanisms to the cartilage destruction that occurs in SA: (1) bacteria and associated toxins, and (2) components of the host immune response. However, the contribution from each of these is currently unknown. This study focuses on the former and aims to provide fundamental information about the effect of *S. aureus* and its toxins on *in situ* chondrocyte viability separate from the potentially confounding effect of the host immune response present in animal models. We have used confocal laser-scanning microscopy (CLSM) to spatially define and quantify *in situ* chondrocyte death in a bovine cartilage model of SA, which is a relatively novel approach. We have also measured cartilage hydration following bacterial exposure to assess cartilage integrity, as increased cartilage water content is a sensitive indicator of cartilage matrix disruption[30,31].

Our primary hypothesis was that both a laboratory strain and clinical isolates of *S. aureus* have a rapid and potent effect on *in situ* chondrocyte viability and cartilage integrity. Our secondary hypothesis was that chondrocyte death commences within the superficial zone (SZ) of cartilage and progresses to deeper layers. This study was conducted to improve our understanding of the interaction between bacteria and *in situ* chondrocytes with a view, ultimately, to improving the treatment of SA.

Materials and methods

Biochemicals and solutions

Biochemicals were obtained from Invitrogen Ltd. (Paisley, UK) unless otherwise stated. The standard tissue culture medium was serum-free Dulbecco's Modified Eagle's Medium (DMEM, 340 mOsm/Kg H_2O; pH7.4) with L-glutamine (4 mM), D-glucose (25 mM) and sodium pyruvate (1 mM). Penicillin (50 U/ml) and streptomycin (50 μg/ml) were added for experiments not involving bacterial culture. DMEM of three pH values was used: pH7.4, pH6.4 and pH5.4 (adjusted using HCl). 5-chloromethylfluorescein diacetate (CMFDA) was prepared in dimethyl sulphoxide (1 mM stock) and propidium iodide (PI) as an aqueous 1 mM stock. Formaldehyde solution (4% v/v in saline; pH7.4) was obtained from Fisher Scientific (Loughborough, UK). The standard bacterial culture media were tryptone soya agar and broth (TSA, TSB; Oxoid Ltd., Basingstoke, UK).

S. aureus strains and isolates

S. aureus strain 8325-4 was kindly provided by Professor T.J. Foster, Dept. Microbiology, Trinity College, Dublin, Ireland. A total of three *S. aureus* clinical isolates [Table I], isolated from joint aspirates of patients presenting with *S. aureus*-induced SA, were obtained. Bacteria were stored at −80°C in 10% v/w skimmed milk (Oxoid Ltd., Basingstoke, UK).

Preparation of defined bacterial aspirates

When required, bacteria were thawed and streaked onto TSA plates and incubated (24 h; 37°C). TSB was then inoculated with several individual bacterial colonies from the TSA plate of a given bacterial strain/isolate and incubated (37°C) in a shaking incubator for 24 h. Serial dilutions, to a maximum of 10^{-6}, were performed on the 24 h TSB culture in order to calculate the number of colony forming units (cfu) in 1 ml of 24 h TSB. Dilutions (100 μl of 10^{-4}, 10^{-5}, and 10^{-6}) were spread onto TSA plates and incubated (37°C; 24 h). Colonies were then determined and counted using a colony counter (Stuart®, Bibby Scientific, Stone, UK). Bacterial counts were performed on a number of cultures and a count of approx. 1.0×10^9 cfu/ml was consistently obtained. The 24 h culture of each strain grown in 10 ml TSB was then diluted in DMEM to produce a final bacterial concentration of approx. 1.0×10^5 cfu/ml DMEM.

Bovine osteochondral explants

Metacarpophalangeal joints of 3-year-old cows were washed, skinned, de-hoofed and opened under sterile conditions within 6 h of slaughter. Only healthy joints, with no evidence of cartilage damage/degeneration, were used. Osteochondral explants were harvested from the convex articular surface[32]. For the experiment investigating the sensitivity of SZ chondrocytes to bacterial toxins, subchondral bone-free explants were obtained.

Bacterial culture and pH studies

For each experiment, osteochondral explants from each joint were placed into separate tissue culture flasks containing DMEM (5 ml). Thereafter, 25 μl (2.5×10^3 cfu) of a given bacterial aspirate was added to each flask. This concentration was chosen as it was in the range used by previous direct joint-inoculation *in vivo* studies of SA[33,34]. In addition, our preliminary experiments identified that this number of bacteria produced a measurable response that was neither overwhelming nor weak. Flasks were then incubated (37°C; 5% CO_2; 40 h). A similar experiment was conducted whereby cartilage explants were cultured in non-infected DMEM at pH values of 7.4, 6.4 or 5.4. For a post-infection long-term chondrocyte viability study, explants were initially cultured with *S. aureus* 8325-4 for 40 h. The infected culture medium was then aspirated and the cartilage rinsed with normal saline (0.9% w/v; Baxter's Healthcare,

Table I
Source and details of *S. aureus* clinical isolates utilised in this study

Isolate	Gender	Age	Source
36V	Male	46	Right hip
28G	Male	74	Right wrist
12R	Male	69	Left knee