The Effect of Proteolytic Enzyme Serratiopeptidase in the Treatment of Experimental Implant-Related Infection

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Background: Infection around an implanted orthopaedic device is a devastating complication, and the treatment of infections involving slime-forming bacteria is especially difficult. The purpose of the present study was to evaluate the effectiveness of a proteolytic enzyme, serratiopeptidase, in the eradication of a periprosthetic infection in an in vivo animal model.

Methods: In sixty Sprague-Dawley rats, the medullary canal of the right femur was drilled through the intercondylar notch and was inoculated with a *Staphylococcus epidermidis* strain (ATCC 35984) with a high slime-producing capacity. The cavity was filled with polymethylmethacrylate cement, and a Kirschner wire that had contact with the knee joint was inserted. None of the animals received any treatment for two weeks. Twenty rats were killed at two weeks after the inoculation in order to determine if the infection had become established. The remaining forty rats were randomized into two groups. One group received serratiopeptidase enzyme injections into the knee joint in addition to antibiotic therapy for four weeks, and the other group received intra-articular saline solution injections together with the same antibiotic therapy. The animals from both groups were killed two weeks after the end of therapy (on Day 56). The knee specimens were evaluated bacteriologically and histologically to determine the prevalence of persistent infection and the effects of the enzyme on local tissue.

Results: At two weeks, inoculated bacteria grew on culture of specimens from twelve (63.2%) of nineteen animals in the no-treatment group. Microbiological testing suggested that infection persisted in only one (5.6%) of eighteen animals in the serratiopeptidase-and-antibiotic group, whereas it was present in six (37.5%) of sixteen animals in the antibiotic-only group ($p = 0.001$). Histological evaluation showed similar results (kappa = 0.92).

Conclusions: Serratiopeptidase was effective for eradicating infection caused by biofilm-forming bacteria in this experimental animal model. The antibiofilm property of the enzyme may enhance antibiotic efficacy in the treatment of staphylococcal infections.

The prevalence of infection after total knee arthroplasty is low, but infection carries catastrophic risks that can lead to additional surgery, loss of the prosthesis, amputation, and even death. The treatment of infection after arthroplasty is controversial[1]. Two-stage reimplantation is currently the most successful treatment, but alternatives include antibiotic suppression therapy, wound débridement, direct exchange, resection arthroplasty, arthrodesis, and amputation[1-5].

Staphylococcus aureus and *Staphylococcus epidermidis* are the pathogens most frequently isolated from the area of an infection at the site of a joint arthroplasty[6-9]. Acute infections are most often caused by *Staphylococcus aureus,* which has a high virulence and the ability to cause suppurative inflammation. Low-virulence microorganisms, especially *Staphylococcus epidermidis,* create subacute infections[7,8,10]. Treatment of these coagulase-negative staphylococcal infections is difficult because of bacterial adherence provided by glycocalyx-enclosed biofilm slimes[11,12]. The biofilm that is formed at the interface between bone and the prosthesis shows resistance to both the host defense mechanisms and antimicrobial therapy[11].

Serratiopeptidase is a proteolytic enzyme derived from the nonpathogenic enterobacteria Serratia E15[13]. It is produced in the intestines of silkworms to break down cocoon walls[13]. This enzyme has been used as an alternative to analgesics and nonsteroidal anti-inflammatory agents[13-15] and also has been used to treat chronic sinusitis and postoperative inflammation[16-19].

The enzyme is believed to induce degradation of insoluble protein products like fibrin, biofilm, and inflammatory mediators. It also reduces the viscosity of exudates, facilitates drainage, and alleviates pain by inhibiting the release of bradykinin, a pain-inducing amine[13,15,20,21]. Increased concentrations of antibiotics (between 1.1 and 8.5-fold) at the site of a gingival infection have been demonstrated in rats in association with the concomitant use of serratiopeptidase[22]. It is thought that enzymatic hydrolysis of the fibrinous coagulum that surrounds bacteria results in the increased antibiotic concentration at the site of infection[22-24]. Among the different proteolytic enzymes that have been investigated, serratiopeptidase has shown the greatest enhancement of antibiotic activity and the greatest inhibition of biofilm formation in vitro[20]. Although serratiopeptidase was shown to enhance the activity of antibiotics against infections with most common pathogens, its effect against periprosthetic infections has not been evaluated in vivo[20,22,25]. The purpose of the present study was to evaluate the effect of serratiopeptidase in the eradication of persistent infection in an animal model that includes periarticular hardware and polymethylmethacrylate cement.

Materials and Methods

Selection of Animals

Sixty Sprague-Dawley rats with a mean weight of 417 g were housed in individual cages with a natural light-dark cycle. This study was approved by our institutional animal care and use committee.

Preparation of Bacteria

A *Staphylococcus epidermidis* strain (ATCC 35984) with a high slime-producing capacity that had been isolated from a patient with an infection at the site of a knee arthroplasty was used for inoculation. This strain of Staphylococcus is ofloxacin-susceptible and coagulase-negative. The organisms were passaged after overnight incubation on blood agar. They were grown for four hours in 20% tryptic soy broth. The bacterial suspensions were washed and resuspended in normal saline solution to a concentration of 1×10^8 colony-forming units (CFU)/mL as determined with absorption spectrophotometry (DigiSpec; Helena Laboratories, Beaumont, Texas). A one-fold dilution (10^7 CFU/mL) was prepared and was stored on ice for fifteen to twenty minutes before application[26,27].

Antibiotic

Ofloxacin was administered subcutaneously at a dosage of 20 mg/kg/day, starting two weeks after inoculation (on Day 14) and continuing for four weeks (until Day 42). Ofloxacin is an antimicrobial agent that has both clinical and experimental applicability against *Staphylococcus epidermidis.* It has been shown not to affect biofilm formation or the adherence of *Staphylococcus epidermidis* to prosthetic devices[28], but it is bactericidal for susceptible strains. Therefore, it was an ideal antibiotic for this experimental study.

Enzyme Preparation

Serratiopeptidase (Protease Type XXVI, product number P27789; Sigma Chemical, St. Louis, Missouri) was dissolved in sterile saline solution and was prepared for each injection at a concentration of 1000 IU/mL after flowing through a 45-μm filter (Syrfil MF).

Experimental Design and Operative Technique

All animals were anesthetized with the administration of ketamine hydrochloride (10 mg/kg) intraperitoneally[29]. The right leg was shaved preoperatively. The skin was cleaned with an iodine solution three times after scrubbing with iodophor for five minutes.

A longitudinal skin incision was made and the knee joint was exposed without the use of a tourniquet. A hand drill with a 1.2-mm-diameter drill-bit was used to penetrate the cortex of the intercondylar notch of the femur into the medullary canal for a depth of 4 cm. The medullary canal was irrigated and suctioned, and 0.01 mL of bacterial suspension (10^7 CFU/mL) was instilled into each cavity. Then, each cavity was filled with polymethylmethacrylate cement and a 1-mm-diameter stainless-steel Kirschner wire was inserted into the cement. The joint remained in contact with the cement and the tip of the wire. The capsule and the skin were closed with 4-0 propylene suture (Ethicon, Bracknell, England).

The animals were examined for clinical signs of infection (swelling and reddening of the knee, loss of passive motion of the knee joint, and weight loss)[9,30]. The body temperature measurements and blood analysis of the animals were done regularly during the experiment[9,26,30].

Therapeutic Studies

Two weeks were allowed to elapse to establish the infection, during which time the animals did not receive antibiotic or injection. Twenty animals (the no-treatment group) were killed two weeks after inoculation (on Day 14) to determine the incidence of infection. The presence of infection was evaluated by means of histopathological and bacteriological evaluations as described below. The remaining animals all received subcutaneous ofloxacin (20 mg/kg/day) for the next four weeks but were divided into two groups of twenty rats each. The rats in Group 1 (the serratiopeptidase-and-antibiotic group) received intra-articular injections of 10 IU of serratiopeptidase solution every day, whereas the rats in Group 2 (the antibiotic-only group) received intra-articular injections of sterile saline solution only. The animals were killed with ether anesthesia two weeks after the end of therapy (on Day 56). Under sterile conditions, the femora were disarticulated at the hip and knee joints and were separated from the surrounding soft tissues. The femora were split longitudinally. One half of each femur was preserved for bacteriological studies, and the other half was used for histopathological evaluation.

Bacteriological Examination

One femoral specimen from each animal was crushed into small pieces under sterile conditions, placed in 5.0 mL of saline solution, and agitated on a vortex mixer for five minutes. Serial dilutions were made, plated on tryptic soy agar, and in-

TABLE I Rate of Infection According to Treatment Group

Treatment Group	Percentage of Rats with Positive Results on Culture*	Percentage of Rats with Negative Results on Culture	Log_{10} CFU/g of Bone†
No treatment	63.2% (12 of 19)	36.8% (7 of 19)	5.35 ± 1.20
Serratiopeptidase and antibiotic	5.6% (1 of 18)	94.4% (17 of 18)	2.92
Antibiotic only	37.5% (6 of 16)	62.5% (10 of 16)	4.04 ± 1.06

*The prevalence of persistent infection was significantly lower in the serratiopeptidase-and-antibiotic group than in the antibiotic-only and no-treatment groups ($p < 0.05$). †The data are given as the mean and the standard deviation. CFU = colony-forming units.

cubated at 37°C for five days. The bacteria were isolated and identified by means of gram stain morphology and biochemical techniques. The cultures were considered to be positive for infection if they demonstrated growth of the bacteria that had been inoculated. Any other bacterial colonization was considered to be contamination and was excluded from the analysis. The results were expressed as the mean (and standard deviation) log_{10} colony-forming units per gram of bone.

Histopathological Evaluation

The second femoral specimen from each animal was fixed in neutral buffered formalin, dissolved of polymethylmethacrylate in chloroform, decalcified, dehydrated, and embedded in paraffin. Five-micrometer-thick sections were prepared, stained with hematoxylin and eosin, and examined with light microscopy. All specimens were examined by the same pathologist (S.S.R.). The specimens were graded for histological evidence of infection severity according to the criteria established by Petty et al.[26].

Statistical Analysis

Statistical analysis was performed with the use of SPSS software (version 10.0; SPSS, Chicago, Illinois). The chi-square and independent-samples t tests were used to identify the differences between treatment methods and outcomes. The agreement between bacteriological and histopathological results was assessed with use of the kappa statistic. The level of significance was set at $p < 0.05$.

Results

There were no signs of infection in the appearance of the wounds, except for slight erythema, during the first two weeks after inoculation. All twenty rats in the serratiopeptidase-and-antibiotic group had slight erythema and mild to moderate swelling of the knee joint. Eight rats in the antibiotic-only group had slight swelling and no erythema, and ten had mild swelling and slight erythema. The other two rats in that group had purulent drainage and died of sepsis in the third

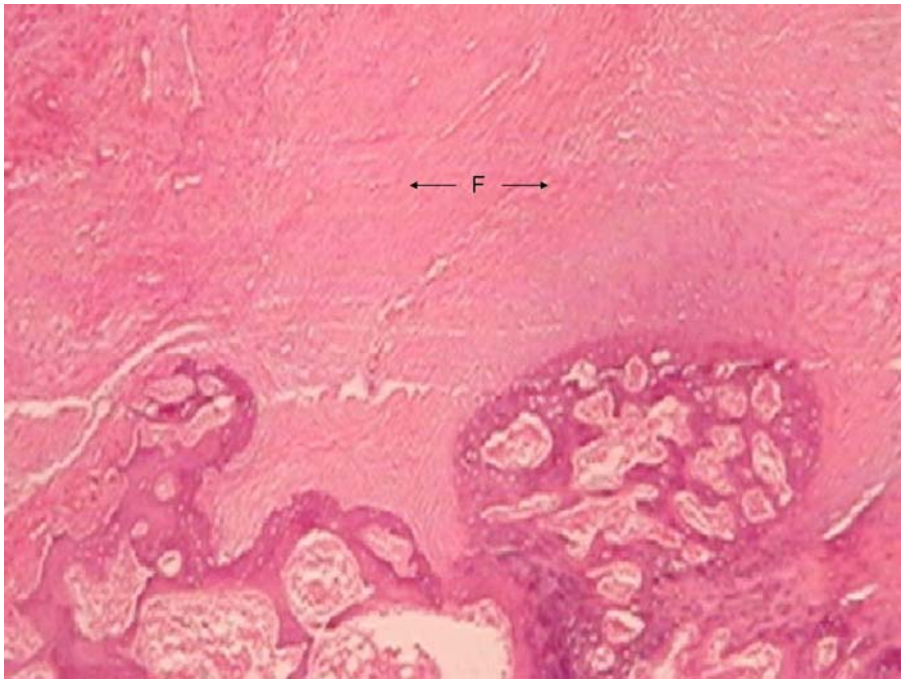

Fig. 1

Photomicrograph of a specimen from the serratiopeptidase-and-antibiotic group, showing abundant fibrosis (F) around the articular cartilage (hematoxylin and eosin, ×100).

TABLE II Classification of Histopathological Findings According to Treatment Group

Grade	No Treatment*	Serratiopeptidase and Antibiotic*	Antibiotic Only*
0 (negligible infection)	7	16	9
1 (minimal infection)	0	0	0
2 (moderate infection)	4	2	6
3 (severe infection)	8	0	1
4 (severe infection)	0	0	0
Total	19	18	16

*The data are given as the number of animals.

week, with blood cultures being positive for the same bacteria that had been inoculated. These two rats were excluded from the study. Brightness of hair was also lost in the antibiotic-only group. The wounds in the serratiopeptidase-and-antibiotic group were mildly warmer than those in the antibiotic-only group. The average body weight in the antibiotic-only group decreased from 417 to 356 g. This weight loss was significantly greater than that in the serratiopeptidase-and-antibiotic group (final mean weight, 406 g; $p < 0.05$). No significant difference was observed between the groups with regard to blood counts or body temperature. No systemic side effects of serratiopeptidase were observed during the study.

Bacteriological Examination

The results of bacteriological cultures are summarized in Table I. At two weeks, the inoculated bacteria grew on culture of specimens from twelve (63.2%) of nineteen animals in the no-treatment group. One animal from this group was excluded from the study because of contamination with *Staphylococcus sciuri.*

On the basis of the microbiological results, infection persisted in only one (5.6%) of eighteen animals in the serratiopeptidase-and-antibiotic group and in six (37.5%) of sixteen animals in the antibiotic-only group ($p = 0.001$) (Table I). With the numbers available, there was no significant difference between the antibiotic-only and no-treatment groups with regard to the rate of persistent infection. Contamination was detected in two cases in each group.

Histopathological Evaluation

Histologically, seven (36.8%) of nineteen animals in the no-

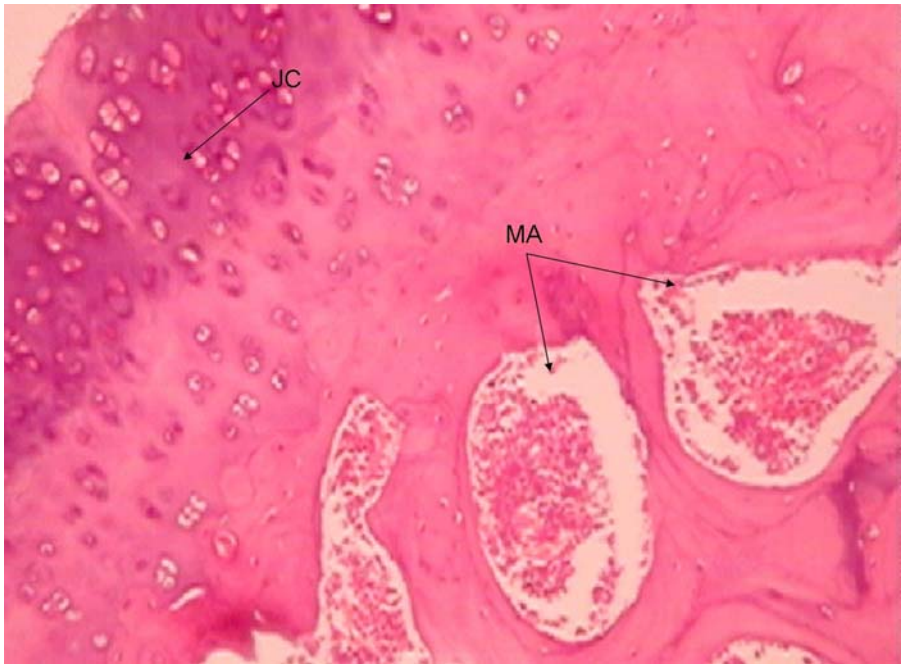

Fig. 2

Photomicrograph of a specimen from the antibiotic-only group, showing joint cartilage (JC) and microabscesses (MA) (hematoxylin and eosin, ×100).

treatment group were classified as having negligible or minimal infection and twelve (63.2%) were classified as having moderate or severe infection. In the serratiopeptidase-and-antibiotic group, sixteen (88.9%) of eighteen animals were classified as having negligible or minimal infection and two (11.1%) were classified as having moderate or severe infection. In the antibiotic-only group, nine (56.3%) of sixteen animals were classified as having negligible or minimal infection and seven (43.8%) were classified as having moderate or severe infection (Table II). On the basis of the histopathological evaluation, there was a significant reduction in the infection rate in the serratiopeptidase-and-antibiotic group compared with the antibiotic-only and no-treatment groups ($p = 0.005$). With the numbers available, there was no significant difference between the antibiotic-only and no-treatment groups.

Histologically, in the serratiopeptidase-and-antibiotic group, polymorphonuclear leukocytes were increased but there was no disintegration in the morphological characteristics of the leukocytes. Microabscess formation was not detected in this group. Granulation tissue and disintegrated polymorphonuclear leukocytes were only present around the drill-hole. New-bone formation was seen along the diaphysis. Articular cartilage was disorganized and thin, with totally destroyed regions. Fibrous tissue was covering the subchondral bone of the metaphysis and the joint capsule, especially around capsular insertions (Fig. 1). Islands of fibrosis were seen in the synovial tissue and the intramedullary endosteal region. In the antibiotic-only group, microabscesses containing disintegrated polymorphonuclear leukocytes and their nuclear debris were seen subchondrally (Fig. 2), subcortically, and subperiosteally along the diaphysis. There was a definite increase in the number of disintegrated polymorphonuclear leukocytes in the medullary canal as compared with the findings in the serratiopeptidase-and-antibiotic group. A positive association was found between bacteriological and histological results (kappa = 0.92).

Discussion

In this experimental model, a cemented Kirschner wire extended from the femur into the knee joint, but gaps in the cement mantle probably provided a pathway for intra-articularly injected bacteria and serratiopeptidase to penetrate the periarticular tissues. Similar penetration was reported in a previous rat model in which an intra-articularly injected high-density polyethylene caused inflammation with a giant-cell response and osteolysis of the bone, resulting in a gap at the bone-cement interface[31].

Fibrinolysis of the slime by the proteolytic enzyme is thought to be the main factor that prolongs the effective period of antibiotic action[23,24]. Treatment of the wound surface with proteolytic enzymes disrupts the coagulum and exposes the bacteria to the antibiotic[23,24]. As a result, there is a decrease in the rate of infection[23,24]. The degree to which enzymes enhance antibiotic action is directly related to the magnitude of their fibrinolytic activity[23,24]. In an in vivo model of vascular graft infection, the combination of intra-abscess urokinase and systemic gentamicin provided significantly better sterilization rates ($p < 0.001$) compared with no treatment and antibiotic treatment alone[32].

Okumura et al. performed a study of patients with osteoarticular infection to examine the concentrations of antibiotic in the venous blood and exudates when serratiopeptidase was administered concomitantly with the antibiotic[33]. The results indicated that the transfer of the antibiotic into the exudate tended to increase in association with the concomitant use of serratiopeptidase.

The persistence of infection in 37.5% of the animals in the antibiotic-only group in the current study was similar to the findings in the study by Petty et al.[34], who reported that infection persisted, in spite of intravenous cefazolin treatment, in 50% to 60% of dog femora that had been inoculated with *Staphylococcus epidermidis*.

In addition to microbiological evaluation, histological examination was performed in the present study. Similar to the findings of Petty et al.[26], there was an excellent but not absolute correlation between the findings on culture and the results of histological evaluation. There are several possible explanations for the discrepancies between the microbiological and histological findings. First, the bacteriological evaluation may have yielded false-negative results for both study groups. Second, in the examination of the histological specimens, it was sometimes difficult to distinguish inflammatory changes associated with the procedure from those caused by infection. This was particularly difficult in cases with minimal or moderate (Grade-1 or 2) inflammation. However, this probably made little difference as statistical tests demonstrated a strong correlation between the bacteriological and histological findings.

The serratiopeptidase-and-antibiotic group showed fibrous tissue, especially at the subchondral bone of the metaphysis, in the synovial tissue, in the capsule, and around the capsular insertion. As no similar finding was present in the antibiotic-only group, the fibrous reaction may have been a direct result of the enzyme. Serratiopeptidase also appeared to have adverse effects on the joint cartilage and the synovial tissue. The joint cartilage was eroded, thinned, and focally destroyed in the serratiopeptidase-and-antibiotic group, but it was intact in the antibiotic-only group. In a previous study, similar adverse effects such as synovitis and cartilage damage were demonstrated after the injection of urokinase directly into the knee joints of healthy mice[35]. That study provided the direct evidence for the inflammatogenic and destructive role of urokinase in vivo. One possible application of combined enzyme-antibiotic therapy is the treatment of infection at the site of a total joint arthroplasty. In that situation, there would be no residual host cartilage remaining, so cartilage damage would not be an issue. Nevertheless, the influence of the enzyme on other periarticular tissues and on long-term implant fixation would still need to be tested in experimental studies. Several studies that have investigated the systemic use of serratiopeptidase have demonstrated no adverse effects[14,16-18,22,33], but there have been two case reports in which serratiopeptidase induced complications[36,37]. In one of those reports, pruritic

erythema with papulovesicles (bullous pemphigoid) developed after the use of serratiopeptidase for the treatment of benign prostatic hyperplasia in a sixty-six-year-old man[36]. In the second case report, acute eosinophilic pneumonia was attributed to serratiopeptidase, which had been prescribed for the treatment of chronic cystitis[37].

Fibrous tissue formation and local inflammatory effects of the enzyme on the capsule, the synovial tissue, and the cartilage are factors that may restrict the clinical use of the enzyme. The dose of the enzyme in the present study was selected empirically on the basis of the study by Selan et al.[20]. The minimum effective serratiopeptidase concentrations that may help to e1radicate infection without causing adverse effects should be investigated. ■

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