In this study, the difference in expression of human beta-defensin-3 in periprosthetic tissue and cancellous bone was observed in the periprosthetic tissue and cancellous bone of patients in the periprosthetic joint infection group, the aseptic loosening group, and the spacer treatment group as well as the synovial membrane and ilium of the normal control group. Hematoxylin and eosin staining of the synovial tissue showed different levels of neutrophil infiltration in all groups except for the normal control group. Immunofluorescence staining of periprosthetic tissue and cancellous bone showed the most positive cells expressing human beta-defensin-3 and the largest mean optical density in the periprosthetic joint infection group, followed by the aseptic loosening group, the spacer treatment group, and the normal control group, with a significant difference in comparison between the periprosthetic joint infection group and the other 3 groups ($P<.01$). White blood cell count, erythrocyte sedimentation rate, and C-reactive protein level were highest in the periprosthetic joint infection group, whereas no difference was found between the other 3 groups. Taken together, high levels of human beta-defensin-3 protein expression were found in the periprosthetic tissue and cancellous bone of patients with periprosthetic joint infection and aseptic loosening, but there are differential expressions of human beta-defensin-3, and this may provide a new marker for the differential diagnosis of infection and loosening of the artificial joint.

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The authors have no relevant financial relationships to disclose.

This study was supported by grants from the Natural Science Foundation of China (30700177, 81071459), Science and Technology Projects of Chongqing (CSTC, 2012gg-yyjs10024), China Postdoctoral Science Foundation (20090460108, 201003775), and Science and Technology Achievements Transformation Fund of Third Military Medical University and Military Twelfth Five Key Projects (BWS11J038).

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Received: June 4, 2013; Accepted: November 22, 2013; Posted: April 15, 2014.

doi: 10.3928/01477447-20140401-61

Human Beta-Defensin-3 for the Diagnosis of Periprosthetic Joint Infection and Loosening

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Artificial joint replacement has been widely used clinically. In 2006, artificial joint replacement was performed in 500,000 cases in the United States and 130,000 cases in the United Kingdom. However, data from multiple centers have shown that the infection rate associated with artificial joint replacement was approximately 1.5% to 2.5% and as high as 9% for elbow joint replacement, and even up to 20% for a revision. The cost for each patient with infection was at least US$30,000 to $50,000. There were 2 million cases of nosocomial infection, including periprosthetic joint infection, in the United States each year, and approximately 50% of these were related to the implant. This complication not only had a cost of up to US$1 billion but also led to serious consequences, such as bacterial resistance, amputation, and even death. Therefore, periprosthetic joint infection has become the most feared complication of joint replacement because of its difficult diagnosis and treatment. Chronic infection caused by bacteria with lower virulence, such as Propionibacterium acnes, is usually difficult to diagnose and differentiate from aseptic loosening. The indicators of this infection, such as neutrophils in the periprosthetic tissue, radionuclide imaging, C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR), have been obtained clinically during surgery. The American Academy of Orthopaedic Surgeons has put forward appropriate diagnostic guidelines and evidence.

As a biologic component of innate immunity, antimicrobial peptides have been found and understood since the 1980s. Antimicrobial peptides are found in a variety of organisms and are considered the future of anti-infective therapy drugs. Antimicrobial peptides have active small-molecule peptides with broad-spectrum antimicrobial activity and are a first line of defense for various living organisms, with the advantages of high efficiency, broad spectrum, and little drug resistance.

On the basis of previous research showing that human beta-defensin-3 had definite inhibition on the formation of Staphylococcus aureus biofilm, the authors studied the differential expression of human beta-defensin-3 in periprosthetic soft tissue and cancellous bone as well as normal synovial tissue and cancellous bone of patients in the periprosthetic joint infection group, the spacer treatment group, and the aseptic loosening group. The study was conducted to examine the relationship between human beta-defensin-3 expression and infection and loosening of the artificial joint and explore its possible use as a new marker and means for the differential diagnosis of periprosthetic joint infection and aseptic loosening to further improve the success rate of artificial joint revision.

**MATERIALS AND METHODS**

**Clinical Materials**

Patient specimens from cases with infection and loosening of artificial joints in the authors’ hospital treated from November 2009 to September 2012 were collected. Periprosthetic tissue and cancellous bone of patients with periprosthetic joint infection (periprosthetic joint infection group, n=26) and aseptic loosening (aseptic loosening group, n=18) were removed during surgery. Two-stage revision and placement of vancomycin-loaded spacers were performed on patients diagnosed with periprosthetic joint infection. Tissue and cancellous bone around the spacers were collected from patients who had undergone reimplantation of an artificial joint after effective control of infection. Knee joint synovium from 13 patients with meniscus injury and hip joint synovium from 2 patients with femoral neck fracture were included as a normal control group of periprosthetic tissue. Normal ilium from patients with limb fracture who had undergone ilium implantation was considered as a normal control group of cancellous bone. Preoperative white blood cell count, ESR, and CRP for all patients were obtained. The study protocol, including access to and the use of the medical records of the study subjects, was reviewed and approved by the institutional ethics committee of Daping Hospital, Third Military Medical University.

Clinical symptoms in the periprosthetic joint infection group included various degrees of persistent pain more than 2 months after the initial replacement. Inclusion criteria were the methods of diagnosing periprosthetic joint infection released by the Musculoskeletal Infection Society in 2012: (a) the sinus tract was connected with the prosthesis; (b) the same pathogens were found in the periprosthetic tissue for 2 or multiple samplings or in bacterial culture of the synovial fluid; and (c) 4 or more of the following 6 criteria were met: (1) increasing CRP and ESR in the serum; (2) increasing white blood cell count in the synovial fluid; (3) an increasing percentage of neutrophils in the synovial fluid; (4) pus in the joints; (5) bacteria found on the 1st bacterial culture of periprosthetic tissue or synovial fluid; and (6) more than 5 neutrophils found in the periprosthetic tissue and at least 5 independent views under a high-power lens (×400). Besides these criteria, infection and loosening needed to be confirmed in all patients through surgery and bacterial culture. This study included 26 patients, including 6 cases of Staphylococcus aureus, 10 of coagulase-negative staphylococci, 4 of Staphylococcus epidermidis, 3 of Pseudomonas aeruginosa, and 3 of uncultured bacteria (Table 1).

**Preparation and Observation of Light Microscopy Samples**

After sterile harvesting of the test specimens, standardized histologic processing was performed. Cancellous bone samples were placed in 0.5 M ethylenediaminetetraacetic acid pH 8.0 buffer solution that was changed weekly. Decalcification was accomplished when the needle could be inserted without resistance. After decalcification and dehydration, the specimen blocks were cut into sections that were 5...
μm thick with an ultra-high-performance microtome for hematoxylin and eosin staining. Histologic morphology and neutrophil infiltration of tissue sections in each group were observed under light microscopy (×200).

**Immunofluorescence Staining**

The sections were dewaxed and rehydrated routinely, and the citrate antigens were cooled to room temperature after thermal repair. Endogenous peroxidase was blocked with 3% hydrogen peroxide and 1% bovine serum albumin. Primary antibody-anti-human beta-defensin-3 (ie, mouse anti-human polyclonal antibody [Novus Biologicals Co, Littleton, Colorado]) was incubated at 4°C overnight. Fluorescent secondary antibody-rabbit anti-mouse fluorescent antibody (Invitrogen Zymed Laboratories, San Francisco, California) and 4′,6-diamidino-2-phenylindole marked with tetramethylrhodamine isothiocyanate dye were incubated and stained with 4′,6-diamidino-2-phenylindole after rewarming. Phosphate-buffered saline was used as the primary antibody for negative control. Staining of positive cells was observed with an inverted-phase contrast fluorescence microscope, and 5 independent views of expressions of positive human beta-defensin cells were selected at random. The mean optical density of each positive cell was measured with Image-Pro Plus 7.0c image analysis software (Media Cybernetics, Bethesda, Maryland).

**Statistical Analysis**

Statistical analysis was performed with SPSS version 17.0 software (SPSS Inc, Chicago, Illinois). The rank-sum test of 2 independent samples was used for comparison between groups. *P*<.05 was considered statistically significant.

**RESULTS**

**Hematoxylin and Eosin Staining of Periprosthetic Tissue and Synovium**

Hematoxylin and eosin staining of periprosthetic tissue and synovium when examined with the naked eye showed that the gross tissue of synovium in the normal control group was soft and bright, but the periprosthetic tissue had a rough texture in other groups. Microscopic observation (×200) with hematoxylin and eosin staining showed dense connective tissue, with no neutrophil or other infiltration in the normal control group, but various degrees of connective tissue, with infiltration of a variable number of neutrophils and lymphocytes in the periprosthetic joint infection group, aseptic loosening group, and spacer treatment group (Figure 1).

**Hematoxylin and Eosin Staining of Cancellous Bone**

Hematoxylin and eosin staining showed no significant difference on gross specimens of cancellous bone in each group when examined with the naked eye. Microscopic observation (×200) of hematoxylin and eosin staining showed no significant difference in neutrophil infiltration in each group (Figure 2).

**Immunofluorescence Staining of Periprosthetic Tissue and Normal Synovium**

After immunofluorescence staining of periprosthetic tissue and normal synovium, an inverted-phase contrast fluorescence microscope (Olympus IX71, Japan) showed that the red particles were positive cells (green arrows) of human beta-defensin-3 proteins stained by tetramethylrhodamine isothiocyanate-marked fluorescent secondary antibody and that the blue particles were nuclei stained by 4′,6-diamidino-2-phenylindole (yellow arrows) (Figure 3). Compared with the aseptic loosening group, the spacer treatment group, and the normal control group, the periprosthetic joint infection group had more positive cells and obviously higher fluorescence intensity. More positive cells and obviously higher fluorescence intensity were shown in the aseptic loosening group than in the spacer treatment group and the normal control group. However, no significant difference was found between the spacer treatment group and the normal control group.

**Immunofluorescence Staining of Cancellous Bone**

The fluorescence microscope (Olympus IX71) showed that the red particles were human beta-defensin-3 proteins in the cytoplasm of the bone cells (green arrows) and the blue particles were nuclei of the bone cells (yellow arrows). Compared with the aseptic loosening group, the spacer treatment group, and the normal control group, the periprosthetic joint infection group had various degrees of higher human beta-defensin-3 protein fluorescence intensity and positive expression in cells in the endosteum (white arrows). More positive expression and obviously higher fluorescence intensity were
observed in the aseptic loosening group than in the other 2 groups. There was a significant difference between the spacer treatment group and the normal control group (Figure 4).

Mean Optical Density of Human Beta-Defensin-3 in Periprosthetic Tissue, Synovium, and Cancellous Bone

Mean optical density of human beta-defensin-3 in the periprosthetic joint infection group was the highest, followed by the aseptic loosening group, the spacer treatment group, and the normal control group. There was a significant difference between the periprosthetic joint infection group.
group and the other 3 groups (P<.01), between the aseptic loosening group and the spacer treatment group and the normal control group (P<.01), and between the spacer treatment group and the normal control group (P<.01) (Table 2). The 95% confidence intervals for the mean optical density of periprosthetic tissue in the periprosthetic joint infection group, the aseptic loosening group, the spacer treatment group, and the normal control group were 0.4253 to 0.4352, 0.3059 to 0.3105, 0.2394 to 0.2440, and 0.2305 to 0.2381, respectively. The 95% confidence intervals for the mean optical density of cancellous bone in the periprosthetic joint infection group, the aseptic loosening group, the spacer treatment group, and the normal control group were 0.4661 to 0.4791, 0.3423 to 0.3480, 0.2394 to 0.2440, and 0.2394 to 0.2440, respectively. The 95% confidence intervals for the mean optical density of cancellous bone in the periprosthetic joint infection group, the aseptic loosening group, the spacer treatment group, and the normal control group were 0.4661 to 0.4791, 0.3423 to 0.3480, 0.2394 to 0.2440, and 0.2394 to 0.2440, respectively.

Discussion

It is not difficult to diagnose acute periprosthetic infection because of its obvious clinical symptoms. Chronic periprosthetic infection is mainly caused by pathogens of lower virulence, such as coagulase-negative staphylococci and P. acnes, and therefore it is difficult to differentiate chronic periprosthetic infection from aseptic loosening. Because there have been no uniform standards for the differential diagnosis of periprosthetic infection, patients diagnosed with aseptic loosening were infected with bacteria of lower virulence and underwent revisions directly because of the positive postoperative culture results. Therefore, the search for new markers to help clinicians to differentiate artificial joint infection from aseptic loosening preoperatively and choose the correct treatment has become a focus in periprosthetic joint infection.

White blood cell count is a commonly used indicator of infection. It increases to varying degrees in patients with local infection, systemic infection, and other noninfectious diseases. However, white blood cell count is not helpful in the diagnosis of periprosthetic joint infection. Di Cesare et al.10 found no statistical significance between the infection group and the noninfection group with regard to white blood cell count preoperatively. The results of the current study also showed no difference in white blood cell count among groups. However, infection is still possible when the white blood cell count increases obviously in patients with acute periprosthetic joint infection.

The most common indicators in the differential diagnosis of periprosthetic joint infection and aseptic loosening are ESR and CRP. Della Valle et al11 reported an accuracy rate of 77% for ESR and 84% for CRP in the diagnosis of periprosthetic joint infection. ESR and CRP still have a high diagnostic efficacy in the diagno-

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Table 2
Mean Optical Density of Human Beta-Defensin-3 in Periprosthetic Tissue and Cancellous Bone

<table>
<thead>
<tr>
<th>Group</th>
<th>Periprosthetic Tissue</th>
<th>Cancellous Bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periprosthetic joint infection</td>
<td>0.43±0.013</td>
<td>0.473±0.017</td>
</tr>
<tr>
<td>Aseptic loosening</td>
<td>0.308±0.005</td>
<td>0.345±0.006</td>
</tr>
<tr>
<td>Spacer treatment</td>
<td>0.234±0.009</td>
<td>0.242±0.005</td>
</tr>
<tr>
<td>Normal control</td>
<td>0.089±0.019</td>
<td>0.138±0.003</td>
</tr>
</tbody>
</table>

*P<.01 compared with periprosthetic joint infection group.
*P<.01 compared with aseptic loosening group.
*P<.01 compared with spacer treatment group.

Table 3
Comparison of White Blood Cell Count, Erythrocyte Sedimentation Rate, and C-reactive Protein

<table>
<thead>
<tr>
<th>Group</th>
<th>White Blood Cell Count, ×10⁶</th>
<th>Erythrocyte Sedimentation Rate, mm/h</th>
<th>C-reactive Protein, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periprosthetic joint infection</td>
<td>6.19±1.98</td>
<td>35.15±23.47</td>
<td>2.39±2.51</td>
</tr>
<tr>
<td>Aseptic loosening</td>
<td>6.15±0.88</td>
<td>8±2.5</td>
<td>0.4±0.09</td>
</tr>
<tr>
<td>Spacer treatment</td>
<td>5.32±1.04</td>
<td>9.83±4.97</td>
<td>0.45±0.23</td>
</tr>
<tr>
<td>Normal control</td>
<td>5.84±1.04</td>
<td>11.07±4.5</td>
<td>0.41±0.09</td>
</tr>
</tbody>
</table>

*P<.05 between all groups.
*P<.05 between aseptic loosening, spacer treatment, and normal control groups.
*P<.01 compared with periprosthetic joint infection group.
sis of periprosthetic joint infection after interference factors are eliminated.\textsuperscript{10,11} Generally, the difference in ESR and CRP between the periprosthetic joint infection group and the aseptic loosening group were statistically significant in this study, but the possibility of periprosthetic joint infection cannot be precluded, even if ESR and CRP are normal, especially in infection caused by bacterium of lower virulence, such as \textit{P} acnes, when both indicators might be normal.

Pathogenic diagnosis is one of the most powerful means for diagnosing infectious diseases. Tissue with suspected periprosthetic infection is often collected during surgery for bacterial culture in the treatment of patients with periprosthetic joint infection. Bacterial culture is always affected by sampling sites, the number of samples, inspection methods, and time limit as well as culture methods and time. Therefore, the bacterium is not necessarily cultured in all of the infected tissue. Most periprosthetic joint infections are chronic infections caused by bacterium of lower virulence and can form biofilms. The bacterium inside the biofilms is dormant and shows atypia,\textsuperscript{12,13} which can lead to false-negative results on bacterial culture.\textsuperscript{14,15} In this study, even if obvious necrosis and pus formation could be seen with the naked eye during surgery, pathogens were cultured from only 20 of 26 infected patients. Negative results on bacterial culture do not necessarily preclude periprosthetic joint infection. However, if the same pathogen is cultured in samples from many sites, this could not only improve the diagnosis rate but also play an important role in guiding the clinical use of antibiotics.

Human defensin is a family of antimicrobial peptides.\textsuperscript{16} Compared with human beta-defensin-1 and human beta-defensin-2, human beta-defensin-3 has stronger antibacterial activity against \textit{Enterococcus faecalis}, such as gram-positive bacteria, gram-negative bacteria, and vancomycin-resistant enterococcus.\textsuperscript{17} Studies have shown that the antibacterial activity of human beta defensins (except for human beta-defensin-3) is strongly inhibited under physiologic salt concentration or in the presence of divalent cations.\textsuperscript{18,19} Human beta-defensin-3 can express at various degrees in the skin, respiratory tract, oral mucosa, trachea, and other tissue\textsuperscript{18} and is related to conditions such as infections and cancer.\textsuperscript{20,21} Warnke et al\textsuperscript{22} found human beta-defensin-3 expression on the endosteum beside the bone trabecula and in the cytoplasm of bone cells rather than in the cytoplasm of bone cells of jawbone under normal conditions. However, there is no finding of similar human beta-defensin-3 expression in periprosthetic tissue and cancellous bone in the periprosthetic joint infection group and the aseptic loosening group.

The mean optical density of human beta-defensin-3 is its expression in periprosthetic tissue of patients with periprosthetic joint infection. In this study, the mean optical density of human beta-defensin-3 in the periprosthetic joint infection group was significantly higher than that in the other groups. The mean optical density of human beta-defensin-3 in the aseptic loosening group was higher than in the normal control group, with statistical difference between groups. This difference might be caused by the local increase and degree of human beta-defensin-3 expression induced by infection or inflammation. Human beta-defensin-3 expression was not found in the normal synovium, but was found in the synovium of patients with osteoarthritis. The normal synovium in the test was obtained from patients with meniscus injury and femoral neck fracture, but the possibility of joint degeneration (ie, osteoarthritis) could not be precluded. In addition, the fluorescence antibody used in the test was tetramethylrhodamine isothiocyanate of red fluorescence that, to some extent, was of autofluorescence. Therefore, the results might not be the same with human beta-defensin-3 expression on normal synovium, and this might be one of the limitations of this study. Paulsen et al\textsuperscript{17} performed experimental studies on 4 groups (10 cases of synovial tissue samples for each group), including patients with pyogenic arthritis, osteoarthritis, and rheumatoid arthritis, as well as a normal control group, using human beta-defensin-1, human beta-defensin-2, human beta-defensin-3, human alpha-defensin-5, and human alpha-defensin-6, respectively, with reverse transcription-polymerase chain reaction. The results showed positive rates of human beta-defensin-3 in the 4 groups of 100%, 100%, 0%, and 0%, respectively. These findings showed that human beta-defensin-3 expression could increase in inflammatory disease of the joints, such as pyogenic arthritis and osteoarthritis. Until now, there has been no research on the expression of human beta-defensin-3 in periprosthetic tissue and cancellous bone in relation to diagnosis and treatment outcomes in patients with periprosthetic joint infection.

Aseptic loosening can also result in inflammation, mainly because of chronic aseptic inflammation caused by stimulation of worn polyethylene particles and other foreign matter.\textsuperscript{23,24} Although infection and aseptic loosening showed different degrees of inflammation in periprosthetic tissue, more neutrophil infiltration was found on frozen pathologic sections during surgery,\textsuperscript{25} indicating that the degree of inflammatory reaction in infected patients was higher than that in patients with aseptic loosening. The mean optical density of human beta-defensin-3 in the infection group showed statistical difference compared with the other 3 groups, especially the aseptic loosening group, which coincided with the results of clinical inflammation and pathologic examination. The infection had been controlled to a certain extent or effectively after comprehensive treatment with debridement, antibiotics, and implantation of spacers for patients in the spacer treatment group.

Thus, the difference between the periprosthetic joint infection group and the
spacer treatment group was statistically significant, suggesting successful infection control. These analyses proved that human beta-defensin-3 expression is associated with the degree of inflammatory reaction of tissue, indicating that human beta-defensin-3 expression in local infected areas might be an indicator that could better reflect the degree of local inflammation. In recent years, studies of the diagnosis of periprosthetic joint infection have focused mainly on local infection.26,27 The tissue in this study, including periprosthetic tissue and cancellous bone belonging to infected local tissue, can reflect the strength of local inflammation. Currently, white blood cell count, ESR, and CRP are commonly used results clinically obtained from peripheral venous blood and reflect systemic reaction of the body against infections and other diseases. These measures may be easily affected by many factors, such as infection and connective tissue in other parts of the body. To make a diagnosis of periprosthetic joint infection, a number of complex systemic and local diseases and other factors that cause increases in white blood cell count, ESR, and CRP must be ruled out. Therefore, human beta-defensin-3 expression, detected partly in this study, can more accurately reflect the degree of inflammation or periprosthetic joint infection, suggesting that higher expression of human beta-defensin-3 also occurs in periprosthetic tissue and cancellous bone as a result of infection or inflammation and correlates with the severity of infection.

**CONCLUSION**

Expression of human beta-defensin-3 can reflect the severity of infection in periprosthetic tissue and cancellous bone of patients with periprosthetic joint infection. Human beta-defensin-3 expression may be a good reference marker for the differential diagnosis of periprosthetic joint infection and aseptic loosening and hence may play an important role in improving the success rate of artificial joint revision.

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