Contribution of reduced interleukin-10 levels to the pathogenesis of osteomyelitis in children with sickle cell disease

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ABSTRACT

Osteomyelitis is a significant complication of sickle cell disease (SCD), and several factors contribute to its pathogenesis, including altered expression of pro-inflammatory and anti-inflammatory cytokines. In view of the role of interleukin-10 (IL-10) as anti-inflammatory cytokine, we tested the notion that SCD osteomyelitis is associated with reduction in IL-10 secretion, and hence precipitation of a pro-inflammatory state.

Study subjects comprised 52 SCD patients with confirmed diagnosis of osteomyelitis, and 165 age- and gender-matched SCD patients with a negative history of osteomyelitis.

Results obtained showed that IL-10 serum levels in SCD osteomyelitis patients were significantly lower than those of control SCD patients. ROC analysis demonstrated that altered IL-10 serum levels predicted the development of osteomyelitis, and the area under ROC curves of IL-10 was 0.810 among SCD patients with osteomyelitis. A systematic shift in IL-10 serum levels toward lower values was seen in osteomyelitis cases, with increased osteomyelitis risk associated with decreased IL-10 levels. Multivariate logistic regression analyses confirmed the independent association of reduced IL-10 with osteomyelitis, after controlling for HbS, HbF, platelet and WBC count. These data support strong association of decreased IL-10 levels with osteomyelitis, thereby supporting a role for IL-10 in osteomyelitis follow-up.

Key words: Inflammation; interleukin-10; osteomyelitis; sickle cell disease
INTRODUCTION

Sickle cell disease (SCD) is an inherited monogenic inherited anemia with variable clinical manifestation and associated disease severity (1) since some patients present with severe complications, while others do not present with any of SCD complications (2). Besides vasoocclusive crisis (VOC), osteomyelitis is a common complication of SCD, and is a major cause for hospitalization of SCD patients (3). It can present as acute or a chronic inflammatory destruction of the bone, and is characterized by progressive destruction of infected bone and recruitment of osteocytes to the infection sites, which include femur, humerus, vertebra, ribs, sternum (4, 5), although any bone may be infected (3, 5, 6). Osteomyelitis is multifactorial in nature, and is influenced by local factors relating to bone lesion and type of infecting microorganism (5, 7), together with inherited predisposition and immunological dysfunction (3, 8, 9).

Distinguishing the acute presentation of osteomyelitis from VOC relies on clinical assessment (fever and pain on admission, swelling of affected limb, and painful sites), radiological findings (ultrasound scans, MRI), in combination with elevated C-reactive protein (CRP) and WBC counts (5, 10). Distinct cytokines profiles were reported during early (1-4 months) and late (5-12 months) osteomyelitis episodes, the former highlighted by increased frequency of high TNFα and IL-4 producers, while the latter is exemplified by increased frequencies of IL-10, IL-6 and IL-2 producers (11). The expression of pro-inflammatory cytokines (IL-1, IL-6, TNFα) was reportedly upregulated in osteomyelitis patients, suggesting an imbalance between pro-inflammatory and anti-inflammatory mechanisms in the pathogenesis of post-traumatic (12) and tuberculosis (13) osteomyelitis.

Functionally, inflammatory cytokines induce osteomyelitis-associated regulation of
osteoclast activity and bone destruction directly (14-17), and/or indirectly through stimulation of
the release of key bone remodeling factors (18). On the other hand, the anti-inflammatory
cytokine IL-4, acting by down-regulating IL-1 and Th1-type cytokines production (19,20), was
described to be involved in the restoration of damaged bone tissue in osteomyelitis (21). Insofar
as the related anti-inflammatory cytokine IL-10 acts as suppressor of infection-stimulated bone
resorption *in vivo* (22) and was associated with SCD complications (23), the aim of this study
was to assess the association between IL-10 serum levels in osteomyelitis in children with SCD.

**SUBJECTS AND METHODS**

**Patients and Controls.** From September 2010 to June 2012, 217 SCD patients (132 males and
85 females), with a mean age of 12.9 ± 8.9 years (range 1 to 17 years), diagnosed according to
hemoglobin profile (HbA, HbS, HbA₂, and HbF) were enrolled. Patients were assigned to one of
two groups: osteomyelitis (n = 52) and no-osteomyelitis SCD patient controls (n = 165),
according to their clinical presentation and laboratory findings. Diagnosis of osteomyelitis was
based on clinical signs, radiological evidence, and laboratory evaluation. Clinical assessment of
osteomyelitis included swelling, warmth, tenderness and inability to bearing weight.
Radiological findings involving plain radiography or MRI were used to rule out other causes. In
addition, confirmatory diagnosis of osteomyelitis was based on hematological investigations, in
particular measurement of ESR and CRP levels. While these lacked specificity, normal ESR and
CRP levels ruled out osteomyelitis. Blood cultures and bone or joint pus aspiration (24), were
also used in diagnosing osteomyelitis; in general a positive blood culture obviated the need for
biopsies. The most common infecting organisms in osteomyelitis patients were salmonella (n =
51; 78.8%) and staphylococcus aureus (n = 11; 19.2%) species, while tibia (38; 64.4%), humerus
(14; 23.7%), and femur (7; 11.9%) were the main sites of infection. Treatment of osteomyelitis consisted of intravenous penicillin and a cephalosporin, used either alone or in combination. Control patients included afebrile SCD patients with no history of SCD-associated bone involvement, and were matched with osteomyelitis patients according to gender, hemoglobin profile (HbS, HbF), and other hematological indices (Table 1). Local research and ethics committees approved the study protocol, which was in agreement with the Helsinki declaration of 2000. All participants (or guardians in the pediatric cases) gave written informed consent, after the purpose of the study was explained to them.

Serum IL-10 Measurement. Peripheral venous blood was collected from osteomyelitis SCD cases during active osteomyelitis episodes, and from steady-state SCD controls into plain tubes (no anticoagulants). Serum was prepared by centrifugation of coagulated peripheral venous blood at 2000g for 10 min at 4°C, and was stored in aliquots at or below -30°C. Samples were tested for IL-10 using human IL-10 sandwich enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN). Assay sensitivity was 3.9 pg/ml, and inter-assay and intra-assay precision (CV%) ranged from 5.9-7.5% and 1.7-5.0%, respectively.

Statistical analyses. Statistical analyses were performed on SPSS version 21 (Statistical Package for the Social Sciences). Categorical variables were expressed as percentages of total, while continuous variables were presented as mean ± SD. Student’s t-test was used to determine differences in means, and Pearson χ² or Fisher’s exact test was used to assess inter–group significance. Utility of IL-10 as predictor of osteomyelitis was examined using receiver-
operating characteristic (ROC) curves. Scatter plot analyses were initially used to present the distribution of IL-10 among groups of individuals, with values out of percentiles 5–95 interval shown as individual points. The osteomyelitis risk was estimated in osteomyelitis patients relative to control SCD patients by calculating the odds ratios (OR) and 95% confidence interval (CI), according to the method of Woolf. IL-10 values were compared using comparison of quartiles technique to detect systematic switch of values toward one of the two groups. OR and 95% CI were also calculated for different cutoff points, based on the distribution in control subjects; IL-10 levels were used as continuous and then as categorized variables.

RESULTS

The demographic and clinical characteristics of the study participants are shown in Table 1. The Osteomyelitis group consisted of 52 SCD patients with confirmed diagnosis of osteomyelitis, while the control group consisted of 165 age- and gender-matched SCD subjects with a negative history of osteomyelitis. With the exception of platelet count (P = 0.039), all biochemical and hematological indices were comparable between the two SCD patient groups. Mean IL-10 serum value in osteomyelitis SCD patients was 15.9 ± 6.4 pg/ml, which was significantly lower than that of control SCD individuals of 27.2 ± 17.8 pg/ml (P <0.001). In addition, there was no association between the nature of the bacterial infection (salmonella vs. staphylococcus) and serum IL-10 levels (P = 0.254).

Receiver operating characteristic (ROC) analysis was performed in order to determine the predictive value of IL-10 levels in the prediction of osteomyelitis (Fig. 1). Area-under-ROC curves provided good discriminatory power for IL-10 and osteomyelitis, and demonstrated that...
altered IL-10 serum levels displayed comparable sensitivities and specificities for predicting the development of osteomyelitis. The Spearman's correlation coefficient between IL-10 and osteomyelitis was -0.308 ($P = 3.2 \times 10^{-6}$) among unselected patients, -0.490 ($P = 3.0 \times 10^{-8}$) in males and -0.414 ($P = 8.1 \times 10^{-5}$) in females. The area under ROC curves of IL-10 was 0.810 (95% CI = 0.742-0.877) among unselected patient, 0.829 ± 0.043 (95% CI = 0.744-0.914) for males, and 0.787 ± 0.059 (95% CI = 0.671-0.903) for females.

A systematic shift in IL-10 serum levels distributions toward lower values was noted in osteomyelitis cases. IL-10 levels were then categorized into 5 strata: percentiles 1-25 (P25), 26-50 (P50), 51-75 (P75), 76-90 (P90) and > 90 (P95), according to IL-10 serum levels present in the control group, and analyzed in regression models, first at the univariate, and later at the multivariate levels. Univariate regression analysis demonstrated a positive dose-effect relationship for IL-10 with osteomyelitis, with increased osteomyelitis risk associated with decreased IL-10 levels (Table 2). The strongest OR was for P90 IL-10 percentiles, where it was associated with a 21.74-fold higher risk than P25 (Table 2).

Given the shift in the IL-10 cutoff values between osteomyelitis and control SCD patients, where significant differences were seen at P25 and >P75 percentile, IL-10 levels in SCD patients were then categorized as per threshold limits corresponding to the low (<18.24 pg/ml), intermediate (18.25-30.00 pg/mL), and high (>30.00 pg/ml) percentiles according to concentrations present in the SCD control group. Setting the lower values as a reference (OR = 1.00), monovariate logistic regression analyses demonstrated a dose-effect relationship for IL-10 with osteomyelitis, with increased osteomyelitis risk seen with decreased IL-10 serum levels (Table 3). Multivariate logistic regression analyses confirmed the independent association of...
reduced IL-10 with osteomyelitis (Table 3). None of the variables entered in the model (HbS, HbF, platelet and WBC count) were found to be associated with osteomyelitis (Table 3).

DISCUSSION

We recently demonstrated a strong association of reduced IL-10 levels and VOC, and its modulation of VOC related parameters in pediatric SCD patients, namely type, frequency, severity and duration of VOC (25). VOC, and associated osteomyelitis is a common acute clinical manifestation in children with SCD. Osteomyelitis presents as acute or chronic infection of the bone, and is characterized by suppurative inflammation, abnormal bone remodeling, together with uncontrolled bone resorption (26). IL-10 acts largely as anti-inflammatory cytokine, and given that inflammatory changes accompany osteomyelitis in SCD, this suggests a role for IL-10 in the pathogenesis of osteomyelitis. To the best of our knowledge, no previous study has examined changes in serum IL-10 levels in SCD osteomyelitis.

VOC and osteomyelitis run a unique clinical presentation in the acute stages of SCD, thereby necessitating careful diagnosis. To investigate the relationship between low IL-10 levels and osteomyelitis in pediatric SCD patients, samples were collected based on clinical assessment and positive radiological findings, along with hematological analysis (erythrocyte sedimentation rate, and C-reactive protein), blood culture and bone or joint pus aspiration, with age- and ethnically-matched pain-free non-osteomyelitis SCD controls during active osteomyelitis episodes, so as to minimize the variability inherent in soluble cytokine determination. Results obtained demonstrated a significant association between reduced IL-10 levels and osteomyelitis.
development, which was confirmed by the enrichment of low IL-10 producers in osteomyelitis cases than in control SCD patients.

The high level of pro-inflammatory cytokines during osteomyelitis can be attributed to frank up-regulation of pro-inflammatory cytokine expression, and/or the absence or low level of anti-inflammatory factors, including IL-10. IL-10, is a Th2 cytokine with immunomodulatory functions and broad spectrum anti-inflammatory activity in various models of infections, inflammation, and some cancers. The immune-inhibitory capacity of IL-10 is largely due to its effect on antigen-presenting cells, and on the production of the Th1 cytokines, notably IL-2 and interferon-γ (IFN-γ), and also to the induction of the Th2 cytokines IL-4 and IL-5 (27-30). The major inflammatory cytokines, IL-1, IL-6, IL-12, and TNF, are dramatically repressed following exposure to IL-10. IL-10 can further inhibit inflammation by increasing the release of IL-1 receptor antagonist by macrophages.

Altered cytokine expression in infected bones, and associated bone resorption and higher osteoclast activities are hallmark features of osteomyelitis (17, 31-34). However, the exact mechanism by which cytokines mediate the changes associated with osteomyelitis are not completely known. Our data support the notion that reduced IL-10 secretion augments inflammatory changes seen in osteomyelitis. While no previous study examined the association of IL-10 with SCD osteomyelitis, recent studies suggest temporal changes in cytokine secretion during the course of osteomyelitis. Early osteomyelitis episodes were accompanied by increased frequency of high TNF-α and IL-4 producers, whereas late osteomyelitis events are characterized by increased frequencies of high IL-4, IL-10, IL-6 and IL-2 producers (11). Reduced IL-10 expression was associated with experimental chronic Staphylococcus aureus-
induced osteomyelitis in a rodent model, and improvement in the course of the osteomyelitis resulting from antibiotic treatment were linked with increased IL-10 expression (35).

Mechanistically, IL-10 suppresses infection-stimulated bone resorption in vivo by inhibiting IL-1α expression, with reduced/absent IL-10 expression associated with a compensatory mechanism involving increased IL-6 secretion, being operational in reducing inflammation (36). IL-10 production is racially restricted, and varies markedly among individuals, thus prompting the speculation that IL-10 low-producers are at higher risk to developing inflammatory diseases. However, in chronic progressive inflammatory conditions, including osteomyelitis, a secondary reactive loss of IL-10 appears rather likely, and thus can not be overlooked.

While our study clearly demonstrated an association between reduced IL-10 secretion and risk osteomyelitis in SCD patients, some limitations to these findings are noteworthy. IL-10 levels were measured following osteomyelitis, which raises the possibility that they were the consequence, but not the cause of osteomyelitis. Another shortcoming was our selection of VOC patients, since only pediatric cases were included, which necessitates performing parallel determinations on adult osteomyelitis SCD patients. Despite these shortcomings, our results support the notion of a covert inflammatory response in osteomyelitis, which will be instrumental in the future management strategies of osteomyelitis episodes.

CONFLICT OF INTEREST DISCLOSURE

The authors declare no competing financial interests.
REFERENCES


FIGURE LEGEND

FIG. 1. ROC curve of IL-10 serum levels for evaluation of osteomyelitis. Spearman's correlation coefficient between IL-10 and osteomyelitis was 0.65 ($P < 0.001$) among unselected patients, and the area under ROC curves of IL-10 was $0.810 \pm 0.034$ (95% CI = 0.742-0.877) among SCD patients.
TABLE 1. Characteristics of Study Participants

<table>
<thead>
<tr>
<th></th>
<th>Osteomyelitis †</th>
<th>Control SCD ‡</th>
<th>P ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>10.1 ± 5.7</td>
<td>12.5 ± 12.8</td>
<td>0.118</td>
</tr>
<tr>
<td>Gender (male:female)</td>
<td>32 : 19</td>
<td>99 : 66</td>
<td>0.268</td>
</tr>
<tr>
<td>Hemoglobin Profile:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbS (%)</td>
<td>71.7 ± 10.9</td>
<td>71.2 ± 8.3</td>
<td>0.800</td>
</tr>
<tr>
<td>HbF (%)</td>
<td>18.1 ± 6.6</td>
<td>19.6 ± 7.6</td>
<td>0.232</td>
</tr>
<tr>
<td>Total hemoglobin (Hb) (g/dL)</td>
<td>9.4 ± 1.2</td>
<td>9.5 ± 1.3</td>
<td>0.252</td>
</tr>
<tr>
<td>Hematological Indices</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC (× 10⁹/L)</td>
<td>9.7 ± 5.0</td>
<td>10.2 ± 4.0</td>
<td>0.417</td>
</tr>
<tr>
<td>Platelets (× 10⁹/L)</td>
<td><strong>388.1 ± 239.8</strong></td>
<td><strong>315.6 ± 172.6</strong></td>
<td><strong>0.039</strong></td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>29.6 ± 4.6</td>
<td>27.7 ± 6.4</td>
<td>0.268</td>
</tr>
<tr>
<td>Mean corpuscular volume (fL)</td>
<td>75.6 ± 9.8</td>
<td>76.6 ± 10.4</td>
<td>0.605</td>
</tr>
<tr>
<td>Mean corpuscular Hb (pg)</td>
<td>25.7 ± 4.0</td>
<td>26.2 ± 5.6</td>
<td>0.573</td>
</tr>
<tr>
<td>Mean corpuscular Hb Conc. (g/dL)</td>
<td>33.6 ± 2.7</td>
<td>34.0 ± 1.6</td>
<td>0.451</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>5.3 ± 3.8</td>
<td>4.9 ± 3.3</td>
<td>0.540</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>15.9 ± 6.4</td>
<td>27.2 ± 17.8</td>
<td><strong>1.3 × 10⁻⁵</strong></td>
</tr>
</tbody>
</table>

† Study subjects comprised 52 SCD patients who had osteomyelitis (Osteomyelitis) and 165 SCD patients who had no history of osteomyelitis (Control SCD). ‡ Pearson’s chi square test (categorical variables), 2-tailed t-test (continuous variables).
<table>
<thead>
<tr>
<th>Cut-off (Percentile)</th>
<th>Osteomyelitis</th>
<th>SCD Controls</th>
<th>P</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>P25</td>
<td>35 (68.6) $^1$</td>
<td>43 (25.6)</td>
<td>$8.4 \times 10^{-5}$</td>
<td>1.00</td>
<td>(Reference)</td>
</tr>
<tr>
<td>P50</td>
<td>8 (15.7)</td>
<td>41 (24.4)</td>
<td>0.001</td>
<td>4.29</td>
<td>1.79 – 10.31</td>
</tr>
<tr>
<td>P75</td>
<td>7 (13.7)</td>
<td>42 (25.0)</td>
<td>0.001</td>
<td>5.03</td>
<td>2.01 – 12.50</td>
</tr>
<tr>
<td>P90</td>
<td>1 (1.9)</td>
<td>26 (15.5)</td>
<td>0.003</td>
<td>21.74</td>
<td>2.82 – 166.67</td>
</tr>
<tr>
<td>P95</td>
<td>0 (0.0)</td>
<td>16 (9.5)</td>
<td>0.002</td>
<td>NA</td>
<td>NA</td>
</tr>
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</table>

$^1$Number of subjects (percent of total)
<table>
<thead>
<tr>
<th>IL-10 Level</th>
<th>Osteomyelitis</th>
<th>SCD Controls</th>
<th>P</th>
<th>aOR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 pg/ml — &lt;18.2 pg/ml</td>
<td>36 (69.2)²</td>
<td>43 (25.6)</td>
<td>4.8 × 10⁻⁵</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>≥ 18.2 pg/ml — &lt;30.0 pg/ml</td>
<td>14 (26.9)</td>
<td>69 (41.1)</td>
<td>0.021</td>
<td>7.47</td>
<td>1.55 – 35.91</td>
</tr>
<tr>
<td>≥ 30.0 pg/ml</td>
<td>2 (3.8)</td>
<td>56 (33.3)</td>
<td>2.7 × 10⁻⁴</td>
<td>17.25</td>
<td>3.72 – 79.99</td>
</tr>
</tbody>
</table>

¹aOR = adjusted odds ratio; covariates that were controlled for were platelet and WBC counts, HbS and HbF levels. ²Number of subjects (percent of total).