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Journal of the Chinese Medical Association 76 (2013) 486–490

www.jcma-online.com

Original Article

# Plasma levels in sepsis patients of annexin A1, lipoxin A4, macrophage inflammatory protein-3a, and neutrophil gelatinase-associated lipocalin

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Received December 12, 2012; accepted January 31, 2013

**Abstract**

*Background*: The relationship between the various cytokine responses that occur during sepsis remains controversial. Emerging evidence indicates that the proinflammatory and anti-inflammatory responses are regulated simultaneously from the beginning of sepsis. However, the roles of the novel anti-inflammatory mediators annexin (Anx)A1 and lipoxin (LX)A4 and the proinflammatory cytokines neutrophil gelatinase-associated lipocalin (NGAL) and macrophage inflammatory protein (MIP)-3a have been studied.
*Methods*: In this study, the plasma levels of AnxA1, LXA4, NGAL, MIP-3a, interleukin (IL)-8 and IL-6 in patients with sepsis were determined on admission to the intensive care unit. The patients were classified into survivors and non-survivors based on their outcome on day 28.
*Results*: AnxA1 and LXA4 levels were decreased in sepsis patients compared with control patients, whereas the levels of the proinflammatory cytokines MIP-3a, NGAL, IL-8, and IL-6 were elevated. Furthermore, a significantly higher level of MIP-3a was detected in nonsurviving patients compared with surviving patients ($p < 0.05$), whereas there were no significant differences between these two groups for the levels of the other mediators. Correlation analysis demonstrated that only NGAL level was closely correlated with the level of IL-6. Univariate analysis indicated that the levels of MIP-3a and IL-8 were independent factors associated with patient survival, but this was not confirmed by the multivariate analysis.
*Conclusion*: AnxA1 and LXA4 plasma levels were found to be decreased in sepsis patients, whereas the levels of MIP-3a and NGAL were found to be elevated. This warrants further study in order to determine the clinical implications of these changes.


*Keywords:* annexin A1; lipoxin A4; macrophage inflammatory protein-3a; neutrophil gelatinase-associated lipocalin; sepsis

## 1. Introduction

Our understanding of the proinflammatory versus anti-inflammatory immune responses during sepsis has rapidly evolved in recent years. Recent studies have demonstrated that proinflammatory and anti-inflammatory responses are regulated simultaneously from the first moments of sepsis and that signs of immunosuppression are also already present during the acute stage of sepsis.[1–5] Proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1α, IL-1β, IL-12, interferon (IFN)-γ, and IL-6, are necessary to initiate an effective inflammatory process in patients with sepsis.[6,7] However, somewhat to the contrary, anti-inflammatory cytokines such as IL-10, IL-13, IL-4, and transforming growth factor (TGF)-β, together with cytokine inhibitors such as soluble TNF receptor (sTNFR)-I, sTNFR-II, and IL-1 receptor antagonist, as well as the soluble IL-1 receptor, are also significantly increased in the circulation of patients with sepsis.[8–12] When the above findings are considered together, it is evident that sepsis does not progress

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1726-4901/$ - see front matter 
http://dx.doi.org/10.1016/j.jcma.2013.05.004

along a preset disease pattern, but needs to be perceived as a highly dynamic biological process. Despite their important role in the pathogenesis of sepsis, the role of cytokines as prognostic factors for the prediction of patient outcome remains to be established. Recent studies have reported that persistently high or increasing levels of proinflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-12) and anti-inflammatory cytokines (IL-10), as well as sTNFR-I, sTNFR-II, IL-1 receptor antagonist, and soluble IL-1γ are found mostly in non-survivors.[13–19] In addition, an early and sustained anti-inflammatory profile, as defined by a high IL-10:TNF-α ratio, has been found to be a compromising factor with respect to the late survival of patients with severe sepsis.[20]

Neutrophil gelatinase-associated lipocalin (NGAL) is an endogenous bacteriostatic protein that is synthesized by renal tubular epithelial cells, neutrophils, and macrophages; furthermore, it is also a novel biomarker for the early diagnosis of acute kidney injury.[21] Macrophage inflammatory protein (MIP)-3a (also designated as chemokine CC ligand 20) is a chemotactic factor that mediates the recruitment of immature dendritic cells, mature dendritic cells, and professional antigen-presenting cells to sites of epithelial inflammation.[22] Annexin (Anx)A1 was originally identified as a corticosteroid-regulated protein, and together with lipoxin (LX)A4, they are both important endogenous anti-inflammatory mediators.[23–25] Both molecules mediate the inhibition of leukocyte trafficking, the stimulation of monocyte migration, and adherence and stimulation of macrophage clearance of apoptotic leukocytes during the resolution phase of inflammation.[26–28] Nevertheless, it is still not clear whether or not the circulating levels of these four novel mediators are able to be used as biomarkers in the clinical setting of sepsis. Therefore, we conducted this study to determine the plasma levels of the two anti-inflammatory mediators AnxA1 and LXA4 as well as various proinflammatory cytokines, namely NGAL, MIP-3a, IL-8 and IL-6, in sepsis patients admitted to an intensive care unit (ICU). These results were then used to explore their prognostic value in terms of predicting patient survival outcome.

## 2. Methods

### 2.1. Patients

This was a prospective, observational study that was performed at a tertiary referral ICU from June 2008 to May 2010. The study was approved by the Ethics Committee of the Taipei Medical University Hospital, and informed consent was obtained from either the patients themselves or their next of kin. Patients were recruited on a consecutive basis. Patients with a diagnosis of sepsis were screened for eligibility. The inclusion criteria consisted of clinical suspicion of infection and evidence of a systemic response to infection; furthermore, these needed to fulfil at least two of four criteria related to the presence of systemic inflammatory response syndrome.[29]

### 2.2. Measurement of cytokines and mediators

Peripheral blood samples were collected from patients on the first day after admission to the ICU. Peripheral blood from 20 healthy persons was also collected as a normal control. Peripheral blood was centrifuged at 250*g* for 10 minutes and subsequently each plasma sample was stored at −80°C until analysis. The levels of cytokines and mediators were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (IL-8, MIP-3a, NGAL, and IL-6: R&D Systems Inc., Minneapolis, MN, USA; LXA4: Neogen Corp., Lexington, KY, USA). The level of AnxA1 was determined as reported by Goulding et al.[30] The limits of sensitivity of the above commercial ELISA kits were as follows: IL-8: <10 pg/mL; MIP-3a: <0.47 pg/mL; NGAL: <0.012 ng/mL; IL-6: < 0.7 pg/mL; and lipoxin A4: <0.01 ng/mL.

### 2.3. Statistical methods

All statistical tests were performed using SPSS for Windows (SPSS Inc., Chicago, IL, USA). The Mann–Whitney nonparametric test was used to compare continuous outcome measures between the patients and controls, and between the survivors and non-survivors. Spearman rank correlation was used for estimating the correlation between variables. Logistic regression was applied for univariate and multivariate analyses to determine the risk factors regarding mortality. Variables with $p < 0.1$ in univariate analyses were entered into multivariate analyses. A $p$ value $< 0.05$ was regarded as statistically significant in two-sided tests.

## 3. Results

### 3.1. Patient characteristics

A total of 66 patients were recruited into this study; the characteristics of these patients are shown in Table 1. The source of sepsis was classified as pulmonary in 57 cases (87%), gastrointestinal in four cases (6%), neurological in four cases (6%), and urosepsis in one case (1%). In total, 27 patients (40%) died within 28 days of admission to the ICU. Further analysis showed that there was no significant difference in the Acute Physiology and Chronic Health Evaluation (APACHE) II score and the use of ventilators between nonsurviving and surviving patients, whereas the nonsurviving patients showed an association with more frequent usage of steroid treatment than the surviving patients (66.7% vs. 36.8%; $p < 0.05$).

The plasma levels of proinflammatory cytokine and anti-inflammatory mediators were examined in these sepsis patients at the time of admission to the ICU. The patients were further classified as survivors and non-survivors based on their outcome on day 28 of the observation process. As shown in Table 2, on admission to the ICU, both surviving and nonsurviving patients showed significantly higher plasma levels of the proinflammatory cytokines IL-8, IL-6, and NGAL compared to the control patients. However, a significant increase in MIP-3a levels over those of the control patients was only observed in

Table 1
Patient characteristics.

| Characteristics | Total ($n = 66$) | Survivors ($n = 39$) | Non-survivors ($n = 27$) | $p$ value* |
|---|---|---|---|---|
| No. | 66 | 39 | 27 | |
| Age (y) | 79 (44–96) | 80.5 (44–96) | 81 (50–95) | 0.99 |
| Male | 50 (75.8) | 30 (76.9) | 20 (74.1) | 0.791 |
| BMI | 21.9 (14.7–52.2) | 20.8 (14.7–52.2) | 22.0 (15.0–31.3) | 0.87 |
| APACHE II | 21.5 (6–37) | 22 (12–37) | 21 (6–35) | 0.2 |
| Ventilator treatment | 46 (70.8) | 26 (68.4) | 20 (74.1) | 0.621 |
| Steroid treatment | 32 (49.2) | 14 (36.8) | 18 (66.7) | 0.018 |
| Hospital days | 25 (4–163) | 32.5 (10–163) | 18 (4–100) | 0.04 |
| White blood cell count[a] | 11,250 (920–29,010) | 11,420 (6070–26,940) | 10,140 (920–29,010) | 0.788 |
| Absolute neutrophil count[a] | 10,058 (660–27,353) | 10,478 (5487–23,815) | 8908 (660–27,353) | 0.421 |
| C-reactive protein (mg/dL)[a] | 9.3 (0.3–27.5) | 9.0 (0.8–27.5) | 12.8 (0.3–22.5) | 0.507 |

Data are presented as $n$ (%) or $n$ (range) unless otherwise identified.
* Survivors versus non-survivors, by Mann–Whitney $U$ test.
APACHE II = Acute Physiology and Chronic Health Evaluation II; BMI = body mass index.
[a] Data are expressed as median (range).

non-survivors. By contrast, the levels of the anti-inflammatory mediators AnxA1 and LXA4 were found to be decreased in both the survivors and non-survivors compared to the control patients. Further investigation demonstrated that there was a significantly higher level of MIP-3a present in nonsurviving patients compared to surviving patients ($p < 0.05$), whereas there were no significant differences in the levels of AnxA1, LXA4, IL-8, NGAL, and IL-6 between the surviving and non-surviving patients (Table 2). Further correlation analysis was performed with respect to white blood cell counts and levels of c-reactive protein, IL-8, IL-6, MIP-3a, and NGAL. Only the serum level of NGAL was found to be closely correlated with the serum level of IL-6 ($r = 0.43$, $p = 0.009$).

Univariate analysis showed that the significant factors related to survival outcome were IL-8 level ($p = 0.008$) and MIP-3a level ($p = 0.021$). However, these two factors were not found to be significant independent factors when a multivariate analysis was carried out (Table 3).

## 4. Discussion

This prospective study investigated the plasma levels of anti-inflammatory and proinflammatory cytokines/mediators in sepsis patients on admission to the ICU. We demonstrated that the plasma levels of the anti-inflammatory mediators AnxA1 and LXA4 were both decreased in sepsis patients compared to normal patients when they were admitted to the ICU. However, the levels of the proinflammatory cytokines, MIP-3a, NGAL, IL-8, and IL-6, were elevated. Our results differ from previous studies in which it has been shown that anti-inflammatory cytokines such as IL-10 are concurrently elevated with proinflammatory cytokines in patients with sepsis.[8,9,11] However, consistent with our results, previous studies have also proposed a biphasic model to explain the pathogenesis of the most severe form of sepsis. In this situation, an initial proinflammatory phase is followed by an anti-inflammatory response (also termed as compensatory anti-inflammatory response syndrome).[31–33] It is not clear why in this study the plasma levels of AnxA1 and LXA4 were lower than the levels found in healthy individuals. One possible explanation is the fact that the majority of our patients were elderly with 79.3% being older than 65 years, and the group as a whole having a median age of 79 years. Furthermore, the control group was not age-matched with the patient group. Therefore, further investigation is needed to explore whether being older is associated with lower plasma level of

Table 2
Plasma levels of anti-inflammatory and proinflammatory cytokines in patients with sepsis.

| Characteristics | Normal | Survivors | Non-survivors | $p$ value* |
|---|---|---|---|---|
| No. | 20 | 39 | 27 | |
| Annexin A1[a] | 4.6 (0.5–14.4) | 4.7 (0.2–38.2) | 1.3 (0.1–42.1) | 1.0 |
| Lipoxin A4[a] | 277.9 (152.0–408.9) | 149.0 (0–325) | 170.5 (3–416) | 0.540 |
| IL-8[b] | 10.6 (4.1–34.4) | 40.6 (3.7–1592.2)** | 127.8 (16.2–2413.9)** | 0.379 |
| MIP-3a[b] | 21.8 (15.3–71.7) | 38.9 (7.1–573.3) | 79.5 (22.0–521.3)** | 0.016 |
| NGAL[a] | 78.4 (53.7–188.3) | 191.8 (7.1–716.8)** | 204.1 (0–878.2)** | 0.918 |
| IL-6[b] | 1.4 (0.9–13.0) | 62.7 (6.5–454.7)** | 127.9 (0–1824.7)** | 0.176 |

Data are presented as median (range).
IL = interleukin; MIP = macrophage inflammatory protein; NGAL = neutrophil gelatinase-associated lipocalin.
*Survivors versus non-survivors, by Mann–Whitney $U$ test.
**Versus normal: $p < 0.01$, by Mann–Whitney $U$ test.
[a] Measured as ng/mL.
[b] Measured as pg/mL.

Table 3
Univariate and multivariate logistic regression analysis of prognostic factors for survival in sepsis patients.

| Variable | Univariate | | Multivariate | |
|---|---|---|---|---|
| | OR (95% CI) | *p* value* | OR (95% CI) | *p* value* |
| Sex, female vs. male | 1.17 (0.37–3.64) | 0.791 | – | – |
| Ventilator treatment, yes vs. no | 1.32 (0.44–3.96) | 0.622 | – | – |
| Steroid treatment, yes vs. no | 3.43 (1.22–9.67) | 0.020 | 3.92 (0.11–136.20) | 0.451 |
| APACHE II (>20 vs. ≤20) | 0.86 (0.30–2.51) | 0.785 | – | – |
| Age (y) (>70 vs. ≤70) | 1.16 (0.38–3.53) | 0.789 | – | – |
| BMI (kg/m$^2$; >20 vs. ≤20) | 2.82 (0.83–9.57) | 0.096 | 13.30 (0.22–818.15) | 0.218 |
| WBC (cells/mm$^3$; >10,000 vs. ≤10,000) | 0.32 (0.10–1.00) | 0.051 | 0.08 (0.01–2.85) | 0.164 |
| CRP (mg/dL; >12 vs. ≤12) | 1.96 (0.64–6.05) | 0.240 | – | – |
| Annexin A1 (>2 vs. ≤2)[a] | 0.53 (0.11–2.62) | 0.438 | – | – |
| Lipoxin A4 (>150 vs. ≤150)[a] | 1.29 (0.39–4.30) | 0.683 | – | – |
| IL-8 (>125 vs. ≤125)[b] | 5.81 (1.60–21.17) | 0.008 | 0.778 (0.02–29.68) | 0.893 |
| MIP-3a (>50 vs. ≤50)[b] | 4.71 (1.27–17.56) | 0.021 | 13.11 (0.45–382.43) | 0.135 |
| NGAL (>200 vs. ≤200)[a] | 1.06 (0.34–3.35) | 0.918 | – | – |
| IL-6 (>70 vs. ≤70)[b] | 2.57 (0.64–10.31) | 0.183 | – | – |

Data are presented as median (range).
APACHE = Acute Physiology and Chronic Health Evaluation II; BMI = body mass index; CRP = C-reactive protein; IL = interleukin; MIP = macrophage inflammatory protein; NGAL = neutrophil gelatinase-associated lipocalin; OR = odds ratio; WBC = white blood cell count.
* †Enrolled for multivariate analysis if $p < 0.05$.
[a] Measured as ng/mL.
[b] Measured as pg/mL.

AnxA1 and LXA4. Such a finding would have implications with respect to elderly patients being more likely to be in an immunocompromised state. Related to the above findings, Kosicka et al reported that the plasma level of AnxA1 is decreased in obese patients and that this contributes to the chronic inflammatory phenotype observed in obesity.[34] The important role of AnxA1 in the normal defense mechanisms was supported by an animal study using AnxA1-null mice, where it was found that these mice were more prone to both acute and chronic inflammatory reactions.[35,36] Taken together, the decrease in the plasma levels of AnxA1 and LXA4 in sepsis patients on admission to ICU suggests that the AnxA1 and LXA4 levels in these sepsis patients are likely to be associated with these patients being in an immunocompromised state.

Consistent with previous studies,[37–39] our results indicate that the proinflammatory cytokines NGAL and MIP-3a are elevated in the peripheral blood of sepsis patients on admission to the ICU; this is in addition to there being increases in IL-8 and IL-6. MIP-3a is responsible for the chemoattraction of immature dendritic cells, effector/memory T cells and B cells to the skin and mucosal surfaces. MIP-3a also plays an important role in the pathogenesis of inflammatory diseases, such as rheumatoid arthritis and cancer.[40] In this study, to the best of our knowledge, we are the first to demonstrate that the level of MIP-3a is elevated in the circulation of sepsis patients on admission to the ICU, and that, in addition to the above, the level of MIP-3a is also significantly higher in patients who died within 28 days of admission to the ICU. This suggests that MIP-3a is likely to be a compromising factor with respect to the late survival of patients with sepsis, although its prognostic value in terms of predicting survival outcome was not supported by the multivariate analysis. Nevertheless, our study had a relatively low number of cases and our findings warrant further study in this area.

Excessive production of proinflammatory cytokines in sepsis patients has been associated with multiple organ-system dysfunction including acute kidney injury.[41] However, at present, there are no biomarkers to predict the development of acute kidney injury before the increase of creatinine level is identified. In this regard, we were interested in establishing whether the plasma level of NGAL was elevated in sepsis patients. Parallel to this, Shapiro et al reported recently that increased plasma NGAL level is a promising novel biomarker for predicting the development of acute kidney injury in patients with sepsis,[42] which suggests that at least some of our patients were at risk of developing acute kidney injury. However, further studies are needed to confirm the role of NGAL level in predicting the development of acute kidney injury in sepsis patients. If confirmed, the finding will help clinicians to design effective treatment strategies that should help to prevent the development of acute kidney injury in sepsis patients.

## Acknowledgments

This study was support by a grant from Taipei Veterans General Hospital (V101C-037). This study was conduct in the Clinical Research Core Laboratory of Taipei Veterans General Hospital. We are indebted to Dr. Ralph Kirby, Department of Life Sciences, National Yang-Ming University, for his help with language editing.

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