The Origin of Multiple Molecular Forms in Urine of HNL/NGAL

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Background and objectives: Several molecular forms of human neutrophil lipocalin/neutrophil gelatinase-associated lipocalin (HNL/NGAL), a novel biomarker for acute kidney injury (AKI), have been found in urine. The origin of these different forms and the effect of antibody configuration on assay performances were investigated in this report.

Design, setting, participants, & measurements: The molecular forms of HNL/NGAL from human neutrophils and present in urine obtained from cardiac surgery patients and patients with urinary tract infection (UTI), as well as secreted from HK-2 cells, were studied by Western blotting. The levels of HNL/NGAL in urine were measured by ELISAs. Kidney injury was simulated by incubation of HK-2 cells under stressful conditions.

Results: The major molecular form of HNL/NGAL secreted by neutrophils is dimeric, whereas the major form secreted by HK-2 cells is monomeric. This was reflected by a predominance of the monomeric form in urine from patients with AKI and the dimeric form in patients with UTIs. The epitope specificities of the antibody used in the ELISAs had a profound effect on assay performance and paralleled differences of the antibodies to identify the different forms of urine HNL/NGAL.

Conclusions: The monomeric form is the predominant form secreted by tubular epithelial cells, and the dimeric form is the predominant form secreted by neutrophils. The development of molecular form-specific assays for HNL/NGAL may be a means to identify the origin of HNL/NGAL in urine and construct more specific tools for the diagnosis of AKI.


Materials and Methods

Urine Samples and Separation on Gel Filtration

Thirty-three urine samples were collected preoperation and at 2 and 24 hours postcardiac surgery. Five urine samples were obtained from patients with proven urinary tract infection (UTI). The samples were}

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immediately centrifuged at 3000 rpm for 15 minutes at 4°C and stored in aliquots at −20°C. The local ethics committee approved the study.

Gel filtration of one urine sample was performed on a Superdex 75 HR 10/30 column using the FPLC system (Amersham Pharmacia Biotech AB, Sweden). Fractions were collected and stored at −20°C. HNL/NGAL was determined using the RIA and ELISAs described below.

**Sensitive ELISAs for HNL/NGAL Quantification**

We established four ELISAs on the basis of different antibodies for HNL/NGAL quantification. In addition, we determined HNL/NGAL concentrations using our previously published monoclonal antibody (mAb)697-polycrclonal antibody-based ELISA (19). The basic protocols for these five ELISAs are the same except for the specific antibodies (Diagnostics Development, Uppsala, Sweden) used in the assay. The performance characteristics of the ELISAs are shown in Table 1.

**Polyclonal Antibody-Based RIA for HNL/NGAL Quantification**

The RIA was performed as described in our previous paper (20).

**Western Blotting**

The neutrophil granule release products were obtained as described (1,20). HK-2-conditioned supernatants at the 72-hour time point were harvested and supplemented with 0.1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, Germany) and Complete protease inhibitor cocktail tablets (Roche, Germany). Nonreducing SDS-PAGE and Western blotting were performed according to the manufacturer’s instructions and our previous paper (19).

**Statistical Analyses**

Statistical analyses were performed by STATISTICA 8.0 (StatSoft) and MedCalc 9.5 (MedCalc Software, Belgium). Values are presented as mean ± SD or median with interquartile ranges. P < 0.05 was considered as significant.

**Results**

**Detection of HNL/NGAL Molecular Forms in Urine by Western Blotting**

One rabbit polyclonal and five mouse mAbs against HNL/NGAL were used to identify the molecular forms of HNL/NGAL present in urines obtained from cardiac surgery patients and patients with UTI. We also detected the HNL/NGAL forms in the neutrophil supernatants. The five mAbs were shown by BIAcore experiments to react to different epitopes. As shown in Figure 1 by two representative urine samples, we found notable differences between the antibodies’ performances. Three major bands were regularly identified by the polyclonal antibodies and were identified as the monomeric and dimeric forms of HNL/NGAL and the complexed, heterodimeric forms of HNL/NGAL. These three forms were also detected by mAb764 and mAb765, but additional bands were also seen with either antibody. However, the polyclonal antibody seemed to have a stronger affinity to the dimer and a weaker affinity to the heterodimer than the two mAbs. mAb764 and mAb765 had very similar performances on detecting all three molecular forms. The affinities of mAb764 and mAb765 to HNL/NGAL from high to low were monomeric, heterodimeric, and dimeric forms, respectively. It is also shown that mAb763, mAb699, and mAb697 have strong affinities to

<table>
<thead>
<tr>
<th>ELISA</th>
<th>Coating antibody</th>
<th>Detecting antibody</th>
<th>Blank</th>
<th>Recovery percent</th>
<th>Intra-assay CV</th>
<th>Serial dilution (%)</th>
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<tbody>
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<td>1</td>
<td>Polyclonal</td>
<td>mAb764</td>
<td>0.055</td>
<td>98.4</td>
<td>1.78</td>
<td>0 to 4.5</td>
</tr>
<tr>
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<td>mAb765</td>
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<td>96.9</td>
<td>3.6</td>
<td>0.6 to 5.5</td>
</tr>
<tr>
<td>3</td>
<td>Polyclonal</td>
<td>mAb697</td>
<td>0.007</td>
<td>92.3 to 100.4</td>
<td>3.1</td>
<td>0.6 to 5.5</td>
</tr>
<tr>
<td>4</td>
<td>Polyclonal</td>
<td>mAb764</td>
<td>0.058</td>
<td>92.5</td>
<td>0.8</td>
<td>0.1 to 5.7</td>
</tr>
<tr>
<td>5</td>
<td>Polyclonal</td>
<td>mAb765</td>
<td>0.058</td>
<td>88.4 to 100.5</td>
<td>2.43</td>
<td>0.1 to 5.7</td>
</tr>
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Table 1. Technology parameters of the HNL/NGAL sandwich ELISAs

The standard ranges of the assays are 0.039 to 5 μg/L. The results of the 1:100 dilutions were set to 100% when the other serial dilutions (1:50 to 1:800) were calculated. The data are shown as the mean or the range of the minimum value and the maximum. CV, coefficient of variation.
heterodimeric forms, whereas the affinities to the dimeric and monomeric forms were weak. However, the ability of the polyclonal antibodies and mAb765 and mAb697 in detecting monomeric and dimeric forms in supernatants of stimulated neutrophil granulocytes seemed very similar. It was also obvious that none of these antibodies detected the heterodimeric forms of HNL/NGAL in the neutrophil supernatants. This was not expected. A possible explanation for this was that matrix metalloproteinase 9 could be degraded by proteases released from the neutrophils, because a broad band at a molecular weight of 60 to 80 kD was detected by these antibodies. Five urine samples obtained from patients with UTI were analyzed by ELISA and Western blotting using the polyclonal antibodies, mAb765, and mAb697. The HNL/NGAL levels ranged from 3.5 to 12 μg/L, with the highest levels found in patient 2 and the lowest levels in patient 5. As is seen in Figure 2, the polyclonal antibody mainly identified a dimeric HNL/NGAL, but also some monomeric and heterodimeric forms. mAb765 showed a similar pattern, whereas mAb697 almost exclusively identified the heterodimeric forms and was only seen in patients 3 and 4.

Performances of Different ELISA Formats for Measuring HNL/NGAL in Urine

We measured the levels of HNL/NGAL in urine obtained preoperation and at 2 and 24 hours postoperation using RIA and five ELISAs that are based on different antibodies (Table 2). The median levels of HNL/NGAL preoperation and 2 hours postoperation measured by RIA were the highest among the six assays. On the other hand, the levels obtained by mAb697-based ELISA 5 were significantly lower than the other assays. Table 2 shows the differences in levels preoperation and 2 hours postoperation as well as overall differences during the 24-hour period. Highly significant differences pre- and postoperatively were seen with all assays. Fold increases of median levels preoperation versus 2 hours postoperation were highest and >70 when measured by ELISA 3 (mAb 764-polyclonal), ELISA 2 (polyclonal-polyclonal) or ELISA 1 (polyclonal-mAb 765), and 30- to 34-fold when measured by RIA, ELISA 4 (mAb 764-mAb 765), or ELISA 5.

Measurements of HNL/NGAL by Different Assays after Gel Filtration of Urine

The following two sets of experiments were undertaken to investigate the performances of the assays in the detection of different forms of HNL/NGAL. Gel filtration of one urine sample at 2 hours postoperation was performed. HNL/NGAL levels in the fractions were measured by the five ELISAs and are shown in Figure 3. Two peaks corresponding to elution volumes of the monomeric and dimeric forms, respectively, were obtained by the five ELISAs. In these experiments, the
heterodimeric forms could not be distinguished from the dimeric forms. The highest levels of HNL/NGAL in peak 2 were obtained with ELISA 1 and ELISA 2. All ELISA assays but ELISA 5 measured similar levels in peak 1 (i.e., dimeric HNL/NGAL [Figure 3 insert]). The ratios between peak 2 (i.e., monomeric) and peak 1 (i.e., dimeric HNL/NGAL) were lowest for ELISA 5 (i.e., 3.0) and highest for ELISA 2 (i.e., 11.3).

The Production of Molecular Forms of HNL/NGAL by Kidney Epithelial Cells

In the supplementary file, we show that HNL/NGAL is produced and secreted by a human kidney epithelial cell line HK-2, mostly in a monomeric form, and that the production and secretion is stimulated by cytokines such as IL-1β and by stressful conditions (supplementary Figures 1 through 3).

Discussion

HNL/NGAL was originally isolated from human neutrophils, and our previous work indicated that the measurement of HNL in blood is a superior means to distinguish acute infections caused by bacteria or viruses (7). However, subsequent studies found that HNL/NGAL may also be expressed in other cells such as in kidney, liver, and epithelial tissue under certain conditions (3,4) and that HNL/NGAL measurement in urine and serum/plasma might serve as a biomarker of AKI (12,13,16–18). Our recent study suggested that the antibody configuration of an HNL/NGAL assay had an effect on the clinical performance of the assay because several forms of HNL/NGAL were identified in urine of patients with AKI (19). In this study, we confirmed and extended these observations. We also show that the monomeric form and to some extent the heterodimeric forms are the predominant forms produced by tubular epithelial cells (supplementary file), whereas the dimeric form seems unique to the neutrophils. The monomeric form was also produced by neutrophils, which was compatible with our previous findings (20). One interesting finding in the study presented here was the difference in recognition of these different forms by our antibodies, because the monomeric and dimeric forms originating from neutrophils were identified by all mAbs and by our polyclonal antibodies. This was contrasted by the almost complete inability of mAb697 to recognize these forms in urine. Also, mAb765 showed a strong reaction to these forms in neutrophil supernatants but only weak recognition of the dimeric form in urine. The reasons for these differences are not clear at this time, but they suggest that there are differences in epitope exposure of the different HNL/NGAL forms and thus differences in molecular structures. These findings may also give a clue to the apparent inability of HNL/NGAL originating from tubular epithelial cells to form dimers. One confounder in the diagnosis of AKI could be UTIs because the presence of neutrophils in the urine might release large amounts of HNL/NGAL (26). Indeed we found elevated levels in such patients. However, Western blotting of these urines showed a predominance of the dimeric form of HNL/NGAL, which is compatible with a neutrophil origin of HNL/NGAL in such cases.

The presence of different molecular forms of HNL/NGAL in
urine and differences in epitope recognition of our antibodies were also reflected by the large differences in HNL/NGAL quantification in urine by our assays. Not only were the pre-operative levels very different despite the same calibrator used in the assays, but also the relative changes after operation. It is apparent that the fold increases were highest with the ELISA that used polyclonal antibodies alone or polyclonal antibodies in combination with either mAb764 or mAb765. These two mAbs were also those that recognized most forms in urine using Western blotting. However, the combination of these two mAbs recognized less, which suggests that additional molecular forms were picked up by our polyclonal antibodies. From our gel filtration experiments, it seems as if these differences in recognition of various forms are primarily related to differences in recognition of the monomeric HNL/NGAL because only one assay seemed to recognize the dimeric form differently. The differences could not be explained by overall analytical performances of the assays because all showed similar sensitivities, imprecision, recovery, etc.

On the basis of fractional excretion of HNL/NGAL in humans (C_HNL/NGAL/C_GFR), in situ hybridization in mice, and the fact that HNL/NGAL is an acute-phase protein, two reports claimed that HNL/NGAL accumulation in urine might derive from local renal synthesis, which comprises the major fraction of urinary HNL/NGAL, and distant organs and immune cells(27,28). In agreement with published data (27–29), our findings support the notion that human tubular epithelial cells have the capacity to produce HNL/NGAL because the mRNA expression and protein production were induced by several conditions relevant to a kidney living under stressful or inflammatory conditions such as is seen during extracorporeal circulation (supplementary file). We found the cytokine IL-1β to be the most potent stimulus, which is compatible with the work of others using a lung epithelial cell line (A549) (30). High levels of neutrophil secretory proteins and cytokines such as IL-1β and TNF-α have been observed during and after cardiac surgery by many previous studies (31,32).

We conclude that HNL/NGAL is present in urine in many different forms and that any development of an assay for the quantification of HNL/NGAL in urine has to take this diversity into consideration. Our results also suggest that it should be possible to construct an assay that preferentially identifies HNL/NGAL originating from the tubular epithelium or the neutrophils because the molecular structure of HNL/NGAL seems slightly different from these two sources. Such an assay should be more specific and sensitive in the detection of AKI and of major benefit to our patients at risk of developing impaired kidney function. This should also provide us with some insight into the mechanisms involved in the development of AKI, some of which may involve the activity of early infiltrating neutrophils (33).

Acknowledgments

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Disclosures

Drs. Venge and Xu hold a worldwide patent of the measurements of HNL/NGAL in human disease. The U.S. patent includes the measurement of HNL/NGAL in inflammation in humans.

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