Clinical significance of urinary ferritin excretion in patients with transitional cell carcinoma

Nan-Haw CHOW1, Chih-Jen CHANG2, Pi-Er CHENG3, Tzong-Shin TZAI4, Chun-Mei HUANG3 and Johnny Shinn-Nan LIN1

1Department of Pathology, 2Department of Family Medicine, and 4Department of Urology, Medical College, National Cheng Kung University, Tainan, Taiwan, Republic of China, and 3Department of Statistics, National Cheng Kung University, Tainan, Taiwan, Republic of China

(Received 20 September 1994/10 February 1995; accepted 28 February 1995)

INTRODUCTION

Ferritin (FRN) is an intracellular protein involved in the sequestration and storage of iron. It is mainly found in the liver, spleen and bone marrow. Measurement of FRN in serum provides a valuable index of the body iron store. Moreover, FRN is also found in body fluids and is produced as an acute-phase protein in the inflammatory response [1-2]. Synovial fluid FRN levels are higher in patients with rheumatoid arthritis than in those with osteoarthritis [1]. The concentrations of FRN in pleural fluid are significantly elevated in patients with rheumatoid pleurisy, malignant effusions and empyema [2]. Its positive correlation with lactate dehydrogenase activity and negative association with levels of glucose and complement components highlight its potential with regard to local inflammation [2].

FRN has also been associated with malignancies [3-5]. Increased serum levels of FRN have frequently been observed in patients with neoplastic disease in the absence of iron overload. A positive correlation between FRN serum levels and the advanced stage of cancer has been observed, although its biological role in cancer patients remains unknown.

Serum FRN is mainly derived from mononuclear cells (MCs). Patients with advanced cancer of the breast, ovaries, lungs, colon and oesophagus display elevated levels of FRN on the surface of MCs [6]. Analysis using indirect immunofluorescence has demonstrated a positive correlation between the percentage of ferritin-bearing lymphocytes and clinical stage in patients with head and neck cancers [7]. These results suggest that subpopulations of tumour-infiltrating mononuclear cells may bear FRN, and contribute to the elevation of serum levels. The cellular infiltrates

Key words: ferritin, inflammation, transitional cell carcinoma, tumour-infiltrating lymphocytes, urological disease.

Abbreviations: ANOVA, analysis of variance; Cr, creatinine; FRN, ferritin; IL-2, interleukin 2; NK, natural killer; MC, mononuclear cell; TIL, tumour-infiltrating lymphocyte; TCC, transitional cell carcinoma.

Correspondence: Dr Nan-Haw Chow, Department of Pathology, National Cheng Kung University Hospital, 138 Sheng-Li Road, Tainan, Taiwan 704, Republic of China.
in, or around, the tumour stroma are collectively designated tumour-infiltrating lymphocytes (TILs). These constitute a mixed population, with the majority being T-cells, while macrophages, natural killer (NK) cells, lymphokine-activated killer cells, eosinophils and neutrophils are present in various proportions [8]. The presence of TILs has been assumed to be an expression of local host response to tumours. The more TILs present in the tumour, the higher the probability of a relatively favourable prognosis for the patient [9].

The measurement of urinary FRN has been suggested to be useful in the diagnosis of transitional cell carcinoma (TCC) [10-13]. A striking reduction in urinary FRN levels after local resection of tumour was observed in a young patient with bladder carcinoma [10]. Tang et al. [11] also showed that urinary FRN concentration is higher in patients with bladder carcinoma than in control subjects, and is helpful in discriminating well-differentiated tumours from poorly differentiated ones. In addition, serum and urine FRN have been suggested to have potential as tumour markers for bladder cancer [12]. About 72% of patients with bladder cancer have been found to have increased levels of FRN in their urine [13]. Detailed information regarding the basis of the measurement remains uncertain.

In this study, a comprehensive evaluation of patients with inflammatory and neoplastic urological diseases was performed. Urinary FRN levels were analysed in relation to the severity of tissue inflammation and parameters of the neoplasm to clarify the source of urinary ferritin and its possible clinical implications.

Preliminary data were presented at the 46th National Meeting of the American Association for Clinical Chemistry, 17-21 July 1994, New Orleans, LA, U.S.A.

**MATERIALS AND METHODS**

**Collection and preparation of samples**

Fresh, randomly collected urine samples from study subjects were evaluated in the National Cheng Kung University Hospital, Tainan, Taiwan. The urine supernatant was collected after centrifugation and stored immediately at -70°C until assay. Each sample was thawed before analysis within 4 months of storage. The analysis included three groups of subjects with urological disease (Table 1). They consisted of 58 freshly diagnosed TCC and 37 patients who were tumour-free and with no evidence of infection as determined by urine analysis. Forty-five patients had inflammatory urological diseases: cystitis (n=15), urinary lithiasis (n=20) and benign prostatic hypertrophy (n=10). Fifty-eight age- and sex-matched normal individuals were included as the control group.

**Auxiliary procedure**

Urinary ferritin levels were measured with a two-site (sandwich) radioimmunoassay (Nichols Institute Diagnostics, CA, U.S.A.) by using two monoclonal antibodies recognizing distinct epitopes on the ferritin molecule. Ab(1) was coupled to biotin, while Ab(2) was radiolabelled for detection. Human liver ferritin in protein matrix was prepared as standard with the assay kit. The samples were first incubated with 125I-labelled Ab(2) in test tubes. Then Ab(1) bound to an avidin-coated bead was added to each tube as a solid phase. The tubes were placed on a horizontal rotator and rotated at 170 rev/min at room temperature for 2 h. After washing the unbound material thoroughly, the radioactivity bound to the bead was measured using a gamma-counter. Creatinine was assayed for each aliquot of urine (Synchron CX3 autoanalyser, Beckman Instruments, Brea, CA, U.S.A.). Relative urinary FRN levels were expressed as a ratio of FRN to creatinine (ng/mg creatinine, Cr), and the serum FRN levels were expressed as ng/ml.

**Histological evaluation**

Histological sections of primary tumours (n=58) from transurethral biopsies or radical operations, removed for diagnostic purposes, were stained with haematoxylin and eosin. Tumour grade and tumour-node-metastasis were examined according to the WHO classification and the UICC system respectively. The degree of lymphocytic infiltration was divided into grade 1 (n=37), grade 2 (n=14) and grade 3 (n=7) categories as described previously [14]. Briefly, only cells around blood vessels in the peri- or intratumoral area of tumours and those cells surrounding the invasive margin of tumours were scored. Polymorphonuclear leucocytes and plasma cells were excluded from the assessment. When moderate cell reaction was only regionally present, the degree of infiltration of the tumours was classified as grade 1.

|**Table 1. Urinary ferritin excretion in patients with each type of urological disease.**
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Category</td>
<td>No.</td>
<td>Sex (M/F)</td>
<td>Age range (years)</td>
</tr>
<tr>
<td>Normal</td>
<td>58</td>
<td>31/27</td>
<td>40-83 (mean: 65)</td>
</tr>
<tr>
<td>Transitional cell carcinoma</td>
<td>58</td>
<td>35/23</td>
<td>42-86 (mean: 64)</td>
</tr>
<tr>
<td>Haematuria</td>
<td>45</td>
<td>27/18</td>
<td>26-84 (mean: 65)</td>
</tr>
<tr>
<td>Tumour free†</td>
<td>37</td>
<td>22/15</td>
<td>27-88 (mean: 60)</td>
</tr>
</tbody>
</table>
Cytoscopic biopsy specimens from tumour-free urothelium \((n=11)\) and haematuria of non-neoplastic origin \((n=8)\) were obtained for evaluation of tissue inflammatory reaction. For tumour-free cases, the predominant cell infiltrates were lymphocytes and plasma cells, while neutrophils and eosinophils were overwhelming in the haematuria samples. The degree of inflammatory reaction was classified subjectively as grade 1 if mild cellular infiltrates were accompanied by intact mucosa \((n=11)\). Grade 2 reaction was defined as focal necrosis and moderate levels of cellular infiltrates or lymphoid aggregates \((n=4)\). When extensive mucosal necrosis was observed with or without densely packed cellular infiltrates or germinal centre formation, the reaction was classified as being of grade 3 severity \((n=4)\).

### Statistics

Statistical analysis was performed after logarithmic transformation of the relative FRN levels in spot urine. Analysis of variance (ANOVA) was used to evaluate the potential differences between the group mean values of patients with urological disease and normal control subjects, and between the mean value of each tumour parameter in each subgroup. Three-factor ANOVA was used to compare the interaction of tumour parameters with urinary FRN levels. Only those variables with a \(P\)-value \(\leq 0.05\) were considered as significant.

### RESULTS

Excretion of FRN in urine showed no significant difference in normal individuals with regard to age \((P=0.41)\) or sex \((P=0.08)\) (data not shown). Hence, these two factors were not considered in the following analysis. The log means of urinary FRN levels were considerably higher \((P=0.02)\) in patients with urological disease than in normal control subjects (Table 1). The result was also significant by non-parametric statistics \((P=0.01)\). Otherwise, no apparent difference was observed between patients with neoplastic and non-neoplastic urological diseases \((P>0.5)\).
Fig. 1. Association of urinary FRN levels with grade of TILs in tumour stroma. Urinary FRN values showed a positive association with the degree of lymphocytic infiltration in urothelial carcinoma (P = 0.0001).

Fig. 2. Correlation of urinary FRN levels with the severity of tissue inflammation in patients with tumour-free status and haematuria of non-neoplastic aetiology. A significant difference was observed between the group means in those with grade 1 tissue inflammation and those with grade 2 (P = 0.03). Horizontal bar represents mean values of each group.

Fig. 3. Serum FRN values were plotted against urinary FRN concentrations in patients with newly diagnosed TCC. The corresponding correlation coefficients were r = -0.14 (P = 0.62).

DISCUSSION

Our study demonstrated that trace amounts of FRN are present in the urine of normal subjects, but that the level is significantly increased in patients with either inflammatory or neoplastic diseases of the urinary tract. While the level of urinary FRN could reflect the degree of inflammatory reaction in the urinary tract, it cannot be diagnostic of urothelial carcinoma. Of particular interest, however, is the association of urinary FRN levels with the severity of lymphocytic infiltration in primary tumours. Since the degree of infiltration has been reported to have prognostic importance for bladder cancer [14], or the subset of superficial tumours [15], determination of urinary FRN may thus deserve attention.

In normal subjects, renal tubular cells have been suggested to be the origin of FRN in urine [16]. Because nephrectomy does not alter the excretion of FRN in tumour-free patients, it seems that the kidneys are not the major source of urinary FRN in patients with urological diseases. The hypothesis that most urinary FRN may be derived from damaged kidney cells [10] could not be verified in the current analysis. Moreover, there is no correlation between urine and serum FRN levels, and a trivial association of FRN values with tumour parameters. The biological properties of TCC thus have little effect on the excretion of FRN in urine. In contrast, we support the view that local inflammatory reaction in the tissue stroma of the urinary tract may be the major source of the FRN that is excreted in urine.

It is interesting to note that urinary FRN levels correlate well with the degree of lymphocytic infiltration in urothelial carcinoma. Since the scale of local immunological reactivity in tumours has been suggested to have prognostic significance [14, 15], the measurement may have potential as an alternative method of evaluating host anti-tumour reactivity.

In terms of the in vivo relevance of tumour infiltration, however, different conclusions can be
drawn with regard to its biological effects. The surveillance hypothesis states that the immune mechanism can suppress the growth of most tumours and confer protection. This theory prompted animal studies and human trials attempting to augment the immune response to malignancies. In contrast, however, accumulated data also imply that tumours are, to some degree or at some stage, dependent for their growth on a positive immune reaction [17,18]. Support for this hypothesis comes from the demonstration that macrophages secrete basic fibroblast growth factor [19,20], a peptide known to promote angiogenesis in tumours. In other words, local immunological reaction at the tumour site could help, rather than inhibit, the growth of tumours, the so-called immunofacilitation theory [17]. The concept emphasizes the potential 'flip side' of tumour immunity. However, it is not possible in our analysis to address this issue. Rather, our data indicate one possible method, in future, of titrating the immune reaction. The prognostic significance of lymphocytic infiltration could then be determined by a long-term follow-up study.

Moreover, available evidence argues that TILs may have incomplete or abnormal functional capacities [21,22]. Freshly isolated TILs from human tumours are poorly responsive to interleukin 2 (IL-2) or mitogens [21]. Low levels of effector function in terms of binding or killing autologous tumour cells [22] and NK-cell activity have been found, although some activities may be recovered by the addition of IL-2 [22]. The information reported above appears to dispute the immunocompetence of TILs in vivo. To address this question, more investigations are needed. Yet support for our observation derives from the demonstration of increased FRN synthesis after in vitro activation and proliferation of lymphocytes [23]. In this regard, urinary FRN may be a biomarker of the activation and proliferation of local immune cells, if it is released from stimulated cells [23].

In addition, the principal immunocompetent cells in tumour infiltration are monocytes, which, among granulocytes, contain the highest FRN levels [24], and T-helper and suppressor cells, which also contain relatively high levels of FRN compared with other cell subpopulations [25]. In view of the wide variation among individuals, we speculate that FRN levels represent the summation of different constituents of the immune cells. In this context, it may be regarded as a functional marker reflecting the immune competence of patients. Further study is imperative to elucidate its potential role as an effective prognostic predictor.

The observation that urinary FRN tends to be positively associated with increasing age in cancer patients merits comment here. The mechanisms underlying such age-related augmentation of the immune activation marker are currently unknown. While this finding is in contradiction to the fact that immunological functions decline with age, the clinical relevance of this finding can be examined in two ways. If TILs have beneficial effects in cancer patients, older patients would be expected to have a better clinical outcome. In fact, this is not the case. In patients with bladder cancer [26], we have found that older patients have a significantly higher risk of developing recurrence after tumour resection. Accordingly, urinary FRN levels appear to parallel the biological potential of tumours. Our findings may be attributed to the stimulation of tumour growth by the immune reaction [17]. In this regard, our observation seems to support the immunofacilitation hypothesis and provides a possible explanation why advanced age is a poor prognostic indicator for patients with bladder cancer. In any case, a prospective longitudinal study is required to make a firm conclusion.

Of particular interest, however, is the finding of unusually high levels of FRN in the urine of patients classified as tumour free at follow-up. The field change of 'grossly normal' urothelial is a well-recognized phenomenon in patients with TCC, and presents the clinician with a challenge in terms of prevention of new recurrence and/or tumour progression. Recently, we observed an alteration in urinary epidermal growth factor in this group of patients [27]. The phenomenon highlights the biochemical changes involved in the processes of neoplastic transformation. With regard to the immunological reaction, Migliari et al. [28] noted an increased number of T-helper/inducer, suppressor/cytotoxic lymphocytes and monocyte/macrophages within the mildly dysplastic/hyperplastic urothelium. The in vivo appearance of activated immune cells in dysplastic urothelium suggests that immune surveillance does play a role in local defence against neoplastic changes. Accordingly, our data imply that an active immune response may be occurring in the 'grossly normal' urothelium. A longitudinal follow-up is necessary to clarify the possible place of this test in an individualized monitoring programme.

We found that urinary FRN was also elevated in patients with inflammatory disorders, to levels indistinguishable from those found in patients with neoplastic disease. This outcome illustrates the inadequacy of the FRN test alone as a tumour marker. The potential to reflect recurrence is also limited by the insignificant difference between the group means of patients with newly diagnosed tumours and those who are tumour free. Altogether, our data emphasize the potential relevance of the measurement of urinary cytokines or peptides in studying the host response to neoplasm [27,29].

In summary, our data indicate that urinary ferritin levels reflect the degree of inflammatory reaction in the urinary tract. The measurement cannot be diagnostic of urothelial carcinoma. A long-term follow-up study is warranted to clarify the role of the test in forecasting patient prognosis.
ACKNOWLEDGMENTS

We gratefully acknowledge Professor Huan-Yao Lei from the Department of Microbiology and Immunology for his critical review of the manuscript and valuable suggestions. This study was supported in part by research grant NCKUH-83-071 from National Cheng Kung University Hospital, Tainan, and by NSC-83-0412-B-006-074 from National Science Council, Taiwan, Republic of Chine.

REFERENCES

22. Nouri AME, dos Santos AVL, Crosby D, Oliver RTD. Correlation between class 1 antigen expression and the ability to generate tumor infiltrating lymphocytes from bladder tumor biopsy. Br J Cancer 1991; 64: 996-1000.