Urinary Protein Analysis in Pre- and Postoperative Cancer Patients

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Urinary proteins from six patients with esophageal cancer and two with stomach cancer were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Analyses were performed on days –1 to 3, 5, 7, 10, 14, and 21 (or 22) after surgery. The protein patterns were scanned by densitometry and divided into nine fractions. The main proteins in the fractions (Fr.) were identified as follows: immunoglobulin G in Fr. A, Tamm-Horsfall glycoprotein (THP) in Fr. B, transferrin in Fr. C, albumin in Fr. D, α_1 -acid glycoprotein in Fr. E, α_1 -microglobulin in Fr. F, retinol binding protein in Fr. G, and β_2 -microglobulin in Fr. I. The protein in Fr. H was not identified. The percentage of each fraction

was calculated from the densitometry pattern of each lane. The percentage values were averaged among all the patients, and pre- and postoperative data were compared. The percentage of Frs. E, F, and G increased on days 1–7, and the changes in these three proteins were similar to changes in serum C-reactive protein (CRP). In particular, the percentage of Fr. G peaked within 1 day of operation, which was faster than for CRP. Conversely, other fractions decreased. These results suggest that urinary protein analysis is useful for monitoring the response to surgical stress. *J. Clin. Lab. Anal.* 19:253–259, 2005. © 2005 Wiley-Liss, Inc.

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INTRODUCTION

To monitor the condition of patients before and after surgery, blood is most often collected from the patients. Routinely monitored parameters of tissue injury or infection include the acute phase proteins in the blood, such as C-reactive protein (CRP) and α_1 -acid glycoprotein (α_1 -AG) (1–8). However, the need to collect blood over long periods places a large burden on the patient. In contrast, urine is obtainable noninvasively. Because most of the proteins in urine are derived from blood plasma (9), changes in the urinary proteins may reflect changes in the blood proteins. In addition, we believe that renal dysfunction is caused by postoperative administration of exogenous fluids and drugs, and that early glomerular and tubule dysfunction cannot be

detected by conventional serum analysis. Therefore, we examined whether the information obtained from urine is sufficient for postoperative monitoring.

Qualitative and quantitative changes in the urinary protein composition, as detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), often precede increased protein excretion (9–13). Current sensitive silver-staining methods allow

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SDS-PAGE analysis of urine proteins without the need for prior concentration steps (13–15).

In this study, we separated urinary proteins in pre- and postoperative samples by SDS-PAGE to demonstrate the complete urinary protein profile and find characteristic protein fractions. Moreover, we investigated whether this urinary protein analysis could provide a means of monitoring the patient's condition, especially with regard to inflammation or renal dysfunction in response to surgical stress.

MATERIALS AND METHODS

Study Subjects

Urine samples were obtained from eight patients (patients 1–8) with esophageal or stomach cancer. Samples were collected before (day–1) and after (days 1–3, 5, 7, 10, 14 and 21 or 22) surgery at the First Department of Surgery, Tokyo Medical and Dental University Hospital. Informed consent was obtained. The patient profiles are summarized in Table 1. None of the patients received antibiotics before the operation, and prophylactic antibiotics were administered after the operation. The urine samples were kept frozen at -80°C and used within 1 month.

SDS-PAGE

Gel preparation and electrophoresis were performed according to the Laemmli method (16). The urine samples were prepared with sample buffer (0.05 M Tris-HCl, 1% SDS, 9% glycerol, 0.9% bromophenol blue, pH 6.8). The urine protein separation was performed with a 4% stacking gel at 15 mA/gel (6×9 cm) for 30 min, followed by electrophoresis on a 12.5% separating gel at 30 mA/gel (6×9 cm) for 40 min. After electrophoresis, the separated proteins in the gels were stained using the Wako Silver Staining kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

The stained gels were analyzed using the NIH image program (developed at the U.S. National Institutes of

Health) and densitometric images were obtained. The densitometric images were divided into nine areas, each of which contained one major band, and the areas were classified as fractions (Frs.) A–I. The percentage of protein in each fraction was calculated from the densitometry pattern of each lane.

A low molecular weight marker (LMW; Amersham Biosciences Corp., Piscataway, NJ) was used for molecular weight estimations.

Measurement of CRP in Sera

Serum CRP was measured by immunoturbidimetry using a biochemical autoanalyzer (model 7070; Hitachi Ltd., Tokyo, Japan).

Western Blotting

Western blotting was performed following the method of Towbin et al. (17). After electrophoresis the proteins were transferred to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA) at 2 mA/cm² for 60 min using a semi-dry Horize Blot AE-6673 (Atto Co., Tokyo, Japan). The membranes were blocked using Block Ace (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) for 30 min at room temperature, then washed three times in dilution buffer (10 mM Tris, 154 mM NaCl, 0.05% Tween 20, pH 7.5) for 10 min at each wash.

The membranes were then immersed in a primary antibody reaction mixture consisting of rabbit anti-human immunoglobulin G (IgG; DakoCytomation, Glostrup, Denmark), rabbit anti-human transferrin (TF; DakoCytomation), rabbit anti-human albumin (ALB; DakoCytomation), rabbit anti-human α_1 -AG (DakoCytomation), rabbit anti-human α_1 -microglobulin (α_1 -m; DakoCytomation), rabbit anti-human β_2 -microglobulin (β_2 -m; DakoCytomation), rabbit anti-human retinal binding protein (RBP; DakoCytomation), or peroxidase-conjugated sheep anti-human uromucoid (Tamm-Horsfall glycoprotein (THP); The Binding Site Ltd., Birmingham, UK) diluted 1:1,000 in dilution buffer, for 60 min at room temperature.

All membranes were then washed three times, for 10 min each, with dilution buffer, and all membranes except the THP membrane were then incubated with peroxidase-conjugated secondary antibody (goat anti-rabbit IgG; Bio-Rad Laboratories, Hercules, CA) diluted 1:1,000 in dilution buffer for 60 min at room temperature. Membranes were then washed three times for 10 min each with dilution buffer. The specific proteins in all of the membranes were detected using a POD immunostain set (Wako Pure Chemical Industries, Ltd.). THP membranes were developed without the addition of secondary antibody.

TABLE 1. Summary of patient profiles

Patient No.	Sex	Age (years)	Cancer location	Operative time (hh:mm)
1	Female	46	Stomach	3:10
2	Male	69	Stomach	3:30
3	Male	56	Esophagus	6:41
4	Male	66	Esophagus	6:40
5	Male	50	Esophagus	4:25
6	Male	69	Esophagus	4:17
7	Male	59	Esophagus	10:00
8	Male	62	Esophagus	6:15

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Wilcoxon's rank-sum test was used to test the significance of differences between pre- and postoperative data. *P*-values less than 0.05 were considered statistically different.

RESULTS

Change in SDS-PAGE Fraction From Day-1 to Day 22

To determine the pre- to postoperative changes in all urinary protein components, we separated urine protein according to molecular weight by SDS-PAGE. Through the pre- to postoperative period, the protein fractions in the urine changed in most of the patients.

An image of the characteristic SDS-PAGE pattern for patient 5 is shown in Fig. 1. The strength of the bands in the staining pattern for patient 5 gradually increased from day 2. In addition, relatively low molecular weight

proteins (approximately 14.4–20.1 kDa) appeared on days 3–7. The staining pattern had nearly returned to that of day-1 on postoperative days 14–22. This result was similar to those obtained in the other patients.

The main band patterns on the SDS-PAGE and densitometric images were confirmed, and divided into nine areas. The bands were identified by Western blotting as IgG (120 kDa, Fr. A), THP (92 kDa, Fr. B), TF (78 kDa, Fr. C), ALB (66 kDa, Fr. D), α_1 -AG (51 kDa, Fr. E), α_1 -m (30 kDa, Fr. F), RBP (25.5 kDa, Fr. G), and β_2 -m (14 kDa, Fr. I). The protein in the 20 kDa (Fr. H) band was not identified.

Changes in Serum CRP and Densitometric Values From Day-1 to Day 22

The changes in mean CRP concentrations in the patients from day-1 to day 22 are shown in Fig. 2a. Preoperative CRP values were in the normal range in six patients ($<0.19 \mu\text{g/ml}$), and elevated in patients 3

Fig. 1. Image of SDS-PAGE and densitometric pattern of proteins in the urine of patient 5. The main separated protein groups on the SDS-PAGE and densitometric images were divided into nine areas (fractions A–I) and the major bands were identified by Western blotting. LMW, low molecular weight marker; IgG, immunoglobulin G; THP, Tamm-Horsfall glycoprotein; TF, transferrin; ALB, albumin; α_1 -AG, α_1 -acid glycoprotein; α_1 -m, α_1 -microglobulin; RBP, retinol binding protein; β_2 -m, β_2 -microglobulin; N.D., not determined.

Fig. 2. Changes in CRP and densitometric values through day -1 to day 22. **a:** Change in CRP in sera. **b:** Changes in densitometric value (mean $\pm$ SEM, * P < 0.05, preoperative vs. postoperative).

(1.95 μ g/ml) and 7 (1.1 μ g/ml). Almost all of the CRP values reached their highest level on postoperative day 2, and then gradually decreased. Significantly increased values were present over days 1–10 (P < 0.05).

Figure 2b shows the changes in the mean densitometric values in the patients from day -1 to day 22. The mean value reached the highest level on day 5 and then gradually decreased. Significantly increased values were present over days 2–7 (P < 0.05).

Change in the Percentage of Each Fraction

Figure 3 shows the postoperative percentage of the fractions relative to the preoperative percentage. The mean preoperative fraction percentages among the patients (Frs. A–C, H, and I) were compared with the postoperative percentages (Fig. 3a). The mean was significantly decreased on days 1–7, with the minimum value occurring on day 2, and gradually increased after that. The minimum values of Frs. A and H, both on day 2, were earlier than those for the other decreased fractions. Fr. I decreased most notably and showed

the lowest value on day 3, as shown in the inset of Fig. 3a.

Compared to the mean preoperative fraction percentage, the postoperative fraction (Frs. E–G) was significantly increased on days 1–7, with the maximum value on day 1, and returned to the preoperative level by day 14 (Fig. 3b). There were significant differences in the individual fraction percentages for Frs. E–G on days 1–7 compared to day -1 (P < 0.05). In particular, fraction G increased dramatically and showed the highest value on day 1, earlier than for the other increased fractions (inset of Fig. 3b).

The changes in ALB in Fr. D were excluded from the analysis because all of the patients received ALB and mineral infusion during postoperative days 0–3.

DISCUSSION

The aim of this study was to verify whether this urinary protein analysis method is a suitable method for monitoring the postoperative condition of patients. In particular, we calculated the percentage of each fraction

Fig. 3. Changes in the percentage of each fraction. The percentage was determined by dividing the whole area of each lane into each fraction area from the densitometry pattern and shown as the changes of postoperative percentage relative to the preoperative percentages. The inset shows the data for all fractions. **a:** Decreased fractions. **b:** Increased fractions (mean $\pm$ SEM, * $P < 0.05$, preoperative vs. postoperative).

seen in the densitometric pattern, and compared them in pre- and postoperative samples to detect surgical stress, such as inflammation or renal dysfunction.

In this study the SDS-PAGE urinary protein pattern intensities rose obviously in almost all patients after their operation. The individual intensities of the protein patterns gradually increased and reached a peak during postoperative days 3–7. Proteins with molecular weights higher and lower than ALB appeared in the postoperative electrophoretic patterns. Therefore, it was considered that some of these patients were suffering renal dysfunction (13,18–21). In addition, they must have suffered surgical stress that altered both glomerular

and tubular function. Moreover, we thought that the patients might have suffered tubular damage from being administered antibiotics after the operation. However, it was considered that the stress was reduced by postoperative days 21 or 22 because the intensities of the patterns on SDS-PAGE had returned to those of day-1 in almost all patients. Figure 2b also shows that a temporary reaction to surgical trauma may have occurred.

The fraction percentages of Fr. A (IgG), Fr. B (THP), Fr. C (TF), Fr. H, and Fr. I (β_2 -m) tended to decrease after the operation. Since the molecular weights of IgG and TF are higher than those of ALB, the relative

excretion of these proteins from the kidney may be less than for proteins smaller than ALB (10–15).

Conversely, the fraction percentages of α_1 -AG (Fr. E), α_1 -m (Fr. F), and RBP (Fr. G) tended to increase after the operation. Since the molecular weights of these proteins are comparatively small, the relative excretion of these proteins from the kidney may be large (10–13). It has been reported that the serum concentration of α_1 -AG can rise to 2 g/L during inflammatory conditions, and α_1 -AG has been shown to accumulate in inflammatory effusions. Its low molecular weight may assist in this (6). The high concentrations of the α_1 -AG fraction we observed are considered to represent overflow from the serum through the kidney.

The percentage of RBP was highest on day 1 and then gradually decreased. Thus, RBP is better as a fast-change parameter than the other proteins we analyzed. The RBP percentage increase is consistent with the reported increase in its rate of excretion in the urine after surgical trauma (22). It has also been reported that retinol-RBP excretion occurs during the acute phase response after surgery (23,24). RBP in serum binds retinol and pre-albumin, and thus minimizes excretion of RBP through the kidney. We speculate that the observed increased RBP in the urine was due to reduced binding to retinol and pre-albumin.

α_1 -m is similar in amino acid sequence and in the locations of introns in its structural gene to α_1 -AG and RBP, these homologous proteins retain some similarity in function at the molecular level (25). We believe that the changes in low molecular weight proteins, including α_1 -AG, α_1 -m and RBP, reflected more quickly the reaction to surgical stress, because most of these proteins are acute phase proteins or indices of nutrition (1–8,26). Furthermore, the phenomenon of febrile proteinuria reported by Stephensen et al. (24) indicates that urinary excretion of low-molecular-mass proteins increases during acute infections. The phenomenon of febrile proteinuria thus suggests a defect in protein reabsorption by the tubular epithelium. The implied lesion may be mediated by inflammatory cytokines, such as interleukin 1, interleukin 6, and tumor necrosis factor, which regulate the acute phase response.

The absolute values of CRP in these patients were rapidly changing, similarly to previous reports (27–29). However, the fraction percentage change of RBP was faster than the concentration change of CRP.

Further studies, including longer follow-ups of clinical data, larger numbers of patients, and more clinical analyses, are needed to define more exactly the predictive ability of these examinations in postoperative patients.

In conclusion, SDS-PAGE is suitable for screening urinary protein fractions to determine which fractions are changing, and to demonstrate the temporary renal dysfunction that can occur after surgery. In addition, urinary proteins, especially RBP, α_1 -AG, and α_1 -m, are valuable for noninvasive monitoring of the response to surgical stress.

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