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Immunoreactive low-molecular-weight epidermal growth factor in urine of patients with renal cell carcinoma*

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Summary. A specific heterogeneous enzyme-linked immunosorbent assay (ELISA) has been established in order to determine levels of low-molecular-weight epidermal growth factor (EGF) in the urine of patients with renal cell carcinoma who had undergone unilateral radical nephrectomy. Urine specimens, i.e., 20 pre- and postsurgical specimens from a group of patients and 22 from a control group, were assayed after the urine had been freed from high-molecular-weight proteins (> 30 kDa) and salts. EGF levels were expressed as urinary EGF/creatinine ratios, and a highly significant decrease ($\alpha = 0.0005$ by Student's *t*-test) of urinary EGF was found in the patient group prior to surgery. The cancer patients also showed an additional loss of urinary EGF after unilateral nephrectomy ($\alpha = 0.0005$ by Student's *t*-test). These data correlate with our previous findings that pro-EGF gene expression is decreased in human renal carcinoma and support the concept that low-molecular-weight urinary EGF is derived from high-molecular-weight kidney pro-EGF.

Key words: Epidermal growth factor (EGF) – ELISA – Urine – Renal cell carcinoma

Malignant growth of cells is caused by a dysfunction of a growth regulatory network, which consists of stimulatory and inhibitory polypeptide growth factors. Epidermal growth factor (EGF) is a regulatory polypeptide with a molecular weight of 6045 (low-molecular-weight EGF, LMW-EGF), which has been postulated to be a crucial factor in carcinogenesis. The highest expression of EGF in humans is in the kidney [9, 23], where it is present as a 145-kDa high-molecular-weight membrane-associated precursor protein (pro-EGF). Recent studies have revealed that LMW-EGF present in urine originates primarily from the kidney [1, 17, 21] and not from plasma. Urinary EGF levels habe been studied in patients with various tumors: EGF output was either increased (carcinoma of the lung, maxilla, esophagus, stomach, thyroid, endometrium and colorectal tract [16, 27, 28] or reduced (bladder and gastric tumors [2, 10]). In non-malignant renal diseases, such as chronic renal failure, glomerulonephritis, diabetic nephropathy or chronic pyelonephritis, a significant decrease of urinary EGF was also observed [4, 11, 15]. Transforming growth factor-α (TGF-α) belongs to the family of EGF-like polypeptides and has been found to be overexpressed in various tumors, including renal cell carcinomas [22]. Its involvement in carcinogenesis has been explained with reference to the high affinity of TGF-α to the EGF-receptor.

Renal cell carcinoma is a relatively rare cancer, that accounts for approximately 3% of all adult cancers, with a male to female ratio of 2:1 and a major occurrence between the fifth and seventh decade of age. The general prognosis of renal cell carcinoma, even after nephrectomy, is poor because about 23% of all patients already have metastasized upon diagnosis. Earlier studies [22] demonstrated that pro-EGF gene expression is dramatically decreased in all renal cell carcinoma patients analyzed. The present study therefore investigates whether this also leads to an alteration of the LMW-EGF level in urine. Levels of immunoreactive LMW-EGF were measured in these patients before and after nephrectomy and compared with those of urine samples from a healthy control group. Since LMW-EGF is known to be the biologically active peptide [7], a separation procedure was utilized prior to the assay in order to eliminate crossreactions with high-molecular-weight forms of EGF (> 30 kDa) which also react with antisera directed against LMW-EGF.

Materials and methods

Preparation of samples

Presurgical as well as postsurgical spot urine samples were collected and stored at −40 °C for subsequent analysis. Postsurgical samples were collected between 1 and 8 days after surgery. The patient group

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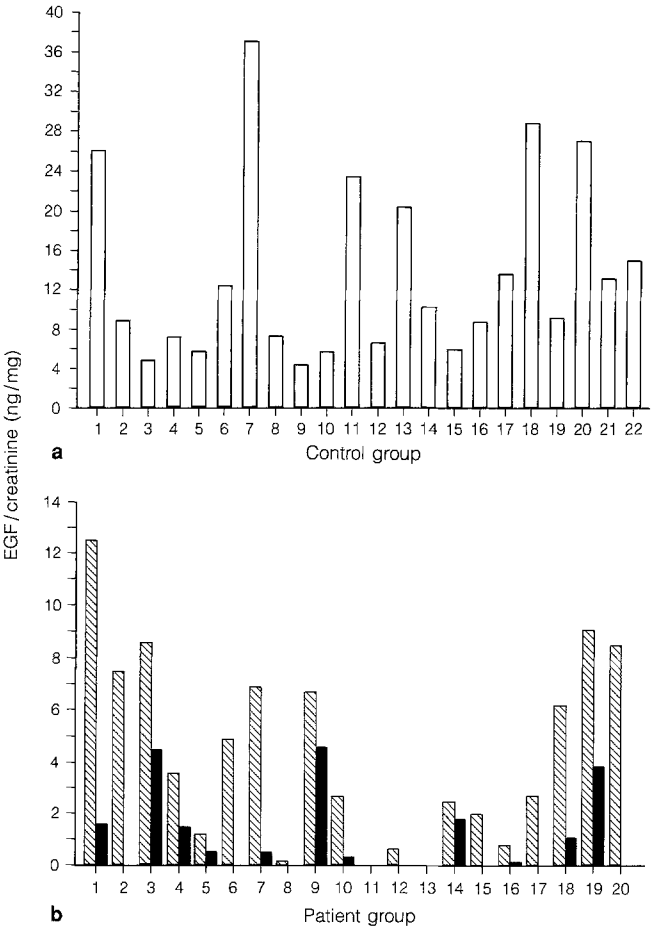

Fig. 1. Levels of LMW-EGF in control individuals and in patients with renal cancer before (▨) and after (■) radical nephrectomy

consisted of 20 subjects (3 women and 17 men) with a median age of 64.45 years (range 38–82 years). All patients were admitted for surgical treatment (radical nephrectomy). The control group consisted of 22 adults (10 women and 12 men) with a median age of 55.7 years (range: 35–76 years). Since high-molecular-weight EGF in urine has been observed in previous studies [7, 18], purification of samples was carried out by centrifugation with centricon-30 microconcentrators (cutoff 30000; Amicon, Witten, FRG). The filtrate was then centrifuged in a centricon-3 microconcentrator (cutoff 3000). The material adsorbed to the membrane was subsequently taken up in carbonate buffer. All samples were assayed in duplicate and in several dilutions in order to rule out influences of the assay by interfering substances.

Antibody production

Peptide conjugation was carried out by mixing 250 µg of human recombinant LMW-EGF (C. George-Nasciemento, Chiron, Emeryville, Calif.) with an equal quantity of keyhole limpet hemocyanin as conjugate [12]. A female New Zealand rabbit was then immunized by s.c. injection of 50 µg of the conjugate in complete Freund's adjuvant at three separate sites. Booster injections were given 15 and 30 days after the initial injection, utilizing incomplete Freund's adjuvant. The serum used in this study was collected 50 days after the initial injection. Preimmune serum was obtained prior to the immunization.

Assay procedures

In order to determine urinary LMW-EGF levels, a heterogeneous enzyme-linked immunosorbent assay (ELISA) was established. Aliquots (100 µl) each sample (preparation as described above) were pipetted into microtiter plates (Nunc, T.C. Interlab, Heidenheim, FRG) and incubated for 60 min at 37°C. Each microtiter plate also contained human recombinant LMW-EGF ranging from 0 to 10 ng/ml over a standard curve. After washing twice with phosphate-buffered saline (PBS) Tween 20, all wells were blocked with a casein hydrolysate (Sigma, Munich, FRG) for 30 min at 37°C. The polyclonal rabbit anti-hEGF antiserum (100 µl) dissolved in PBS/tween 20 (1:500) was then added to each individual well and incubated for 60 min at 37°C. All wells were then washed three times with PBS/Tween 20. As the second antibody an alkaline-phosphatase labelled goat anti-rabbit-IgG antibody (100 µl/well) (Sigma) was incubated for 45 min at 37°C. After additional washing, 100 µl *p*-nitrophenylphosphate dissolved in 0.1 *M* glycine buffer was applied to each well. Dye formation was measured by ultraviolet absorption at 405 nm in an ELISA reader (EAR 400 FW, SLT Laboratory Instruments Austria). For data analysis, the maximum EGF value in the dilution curve of each sample was used. The detection limit of our assay was 1 ng/ml in the final sample.

Controls

Unspecific crossreactions were determined by the use of a preimmune serum from the rabbit, in parallel assays. Incubation with human recombinant TGF-α (R. Derynck, Genentech, San Francisco, Calif., USA) as antigen, in order to rule out crossreactions with EGF-related molecules, confirmed the specifity of the EGF antibody. Using radioactively labelled I^{125}-EGF (192 469 Counts/min) dissolved in bovine serum albumin containing carbonate buffer, the recovery of EGF through the different purification steps was quantified. In order to eliminate the effects of water excretion, urinary EGF levels were expressed as nanograms of EGF per milligram of urinary creatinine. Creatinine concentrations of all urine samples were determined by the kinetic method of Jaffe [13]. Statistical analysis was carried out with Student's *t*-test.

Results

Recovery analysis revealed that, in our assay system 63.7% of the pure recombinant LMW-EGF can be recovered. Therefore, all values measured in this study were corrected by multiplication by 1.57. The material adsorbed to the membrane of the 30-kDa-cutoff centricon microconcentrator also contained EGF immunoreactivity indicating the presence of high-molecular-weight forms of EGF. Figure 1 shows LMW-EGF/creatinine ratios of all control, pre- and postsurgical samples. LMW-EGF was present in all control urine samples analyzed. The median LMW-EGF level of this group was 13.66 ng/mg (range: 4.41–36.94), while LMW-EGF levels in presurgical samples of the patient group had a median value of 4.38 ng/mg (range: 0.00–12.43). This decrease was statistically different at the 0.0005 level. Nephrectomy caused an additional drop of LMW-EGF in postsurgical samples to 1.02 ng/mg (range: 0.00–4.61) (again significant at the 0.0005 level). In eight patients, however, urinary LMW-EGF in postoperative samples were below the detection limit of our assay and in two of these patients urinary

LMW-EGF could not be determined in preoperative samples. Mean concentrations of creatinine in urine were 1.08 mg/ml in the control group, 1.30 mg/ml in preoperative and 0.81 mg/ml in postoperative samples. Since urinary creatinine levels in these three groups were very similar, results can only be due to an alteration of LMW-EGF excretion into urine.

Discussion

A positive correlation between urinary EGF exretion and renal filtration of creatinine has been shown previously [3]. The results of our study show a highly significant reduction of LMW-EGF excretion into urine of patients with renal cell carcinoma, although individual values among patients and control subjects overlap. Hence, the present study failed to show that decreased urinary LMW-EGF is a helpful indicator for the presence or renal cell carcinoma. The drop of LMW-EGF secretion into urine correlates with our previous observation that the expression of the pro-EGF gene is decreased in malignant renal tissue [22]. In addition, in postsurgical urine samples LMW-EGF levels were found to be highly significantly lowered compared with presurgical samples. The latter observation confirms the results of other authors [15, 16, 17, 21], who describe a decrease of urinary EGF after kidney removal in patients and in rats. In our patient group the drop of LMW-EGF concentration in urine exceeded the expected 50% range. This could indicate that, during renal hypertrophy following surgical removal, more LMW-EGF is retained in the remaining kidney and therefore less peptide is secreted into urine. In this context, it is interesting to note that after unilateral nephrectomy in mice, hypertrophy of the remaining kidney is associated with a transient increase of EGF within the renal tissue [8]. Since postoperative samples of our study were collected at different time intervals after surgery, the larger than anticipated drop of LMW-EGF could also be a consequence of a change of the distribution of LMW-EGF between renal tissue and urine during the first postoperative week. In order to address this question, longterm follow-up investigations are necessary. A previous study by Mattila et al. [16], which also analysed eight patients with renal cell carcinoma, did not find a decrease of urinary EGF (determined by radioimmunoassay). The different results of this group can be explained by the purification system that has been utilized for the assay for the present study. By using two membranes with different cutoffs (3 and 30kDa), all samples were freed from potentially interfering salts and the total content of proteins was limited to a range from 3 to 30 kDa. Our assay thus, achieved a relatively high homogeneity of samples and was sensitive only for low-molecular-weight forms of EGF. Although unequivocal results about the age-dependence of EGF excretion into urine are not available, the majority of the investigations indicates that EGF levels are reduced in older people [6, 14, 26, 29]. An age-related loss of urinary-EGF is, however, relevant only until the age of 40, and almost all subjects of this study were older than this. Immunocytochemical investigations in the mouse have shown that EGF is mainly localized at the luminal membrane of the thick ascending limb of Henle as well as at the distal convoluted tubule, whereas the EGF-receptor is immunolocalized at the basolateral and abluminal membrane [5, 19, 20, 24, 25]. This supports the view that LMW-EGF is not the physiological ligand for the EGF-receptor present in renal epithelial cells but may have a target receptor in a different location. High-molecular-weight pro-EGF may possess an additional function related to its association with the cell membrane. In view of the alteration of urinary EGF levels in benign renal diseases, the decrease of LMW-EGF secretion in renal cancer may therefore reflect a general response of renal epithelial cells to injury.

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