Urol Res (1994) 22:221–225

ORIGINAL PAPER

N.H. Chow · T.-S. Tzai · P.-E. Cheng · C.-J. Chang
J. S.-N. Lin · M.-J. Tang

An assessment of immunoreactive epidermal growth factor in urine of patients with urological diseases

Received: 10 November 1993/Accepted: 13 May 1994

Abstract To examine the excretion of urinary epidermal growth factor (EGF) in urological diseases and the relationship of EGF urine levels with transitional cell carcinoma (TCC), we measured the concentration of EGF by radioimmunoassay. The series comprised patients with active TCC ($n=52$), others in tumor-free status ($n=29$) and with non-neoplastic inflammatory diseases ($n=43$), and normal controls ($n=50$). Urinary EGF values were lower in patients with urological diseases of different etiologies than in normal controls ($P<0.005$). Mean EGF levels of patients who had previous bladder tumor resection ($n=21$) were not statistically different from normal controls ($P=0.2$). For patients with active TCC, EGF urine levels showed a significant inverse relationship to increasing tumor grade ($P=0.02$). In addition, subjects who had received nephrectomy for pelvic carcinoma ($n=8$) showed significantly lower mean EGF values than those with intact kidneys ($n=21$), irrespective of sex ($P<0.05$). Immunostaining of EGF on non-neoplastic kidney ($n=9$) revealed reactivity in the distal convoluted tubules and thick ascending limbs of Henle. Our results suggest that the kidney is the major source of urinary EGF. Its excretion in urine is decreased in both inflammatory and neoplastic diseases of the urinary tract. EGF may play an important part in the biological activity of TCC. Further study is indicated to investigate the monitoring of EGF urine levels as a marker of recurrence for EGF receptor-positive TCC.

Key words Urological diseases · Epidermal growth factor · Epidermal growth factor receptor · Transitional cell carcinoma · Nephrectomy · Immunohistochemistry

N.-H. Chow (✉)
Department of Pathology,
National Cheng Kung University Hospital,
138, Sheng-Li Road, Tainan, Taiwan 70428, ROC
Fax: +886(6)2766195

T.-S. Tzai · J. S.-N. Lin
Department of Urology, National Cheng Kung University Hospital,
138, Sheng-Li Road, Tainan, Taiwan 70428, ROC

P.-E. Cheng
Department of Statistics, National Cheng Kung University,
Tainan, Taiwan 70428, ROC

C.-J. Chang
Department of Family Medicine,
National Cheng Kung University Hospital,
138, Sheng-Li Road, Tainan, Taiwan 70428, ROC

M.-J. Tang
Department of Physiology, National Cheng Kung University,
Tainan, Taiwan 70428, ROC

EGF is a regulatory polypeptide that can modulate cellular activity through interactions with its receptor (EGFR) on the cell membrane [2]. In vitro studies have demonstrated its ability in stimulating clonal growth [11, 18], induction of ornithine decarboxylase (ODC) activity [18], and activation of the endogenous tyrosine kinase [1] of bladder cancer cell lines. Since higher EGF values are found in urine than in body fluids, expression of EGFR in bladder cancer might favor tumor proliferation. Clinical studies suggested that invasive bladder tumors tend to express high levels of EGFR [20] and have a poor prognosis [21]. Preliminary studies revealed low urinary EGF levels for patients with active urothelial tumors [5] and those in remission [10]. Fuse et al. [5] demonstrated a decrease of urinary EGF in limited cases of T4 tumors, but no significant association was observed with tumor grade or stage. In contrast, EGF urine levels showed no difference between patients with chronic cystitis and control groups [8]. Accordingly, examination of urinary EGF seems to have the potential to distinguish between the characters of urological diseases.

Excretion of EGF in urine shows a sex difference, and a decline with advancing age [25]. Otherwise, there was no apparent diurnal variation and no influence of hormonal status for women [15]. Several studies have suggested a renal origin for human urinary EGF [16], and this impression is reinforced by a sharp decrease in EGF following unilateral nephrectomy [4]. There is, however,

Table 1 Urinary epidermal growth factor levels in each group of patients with urological diseases

Category	No.	Sex (M/F)	Age range (years)	EGF excretion (ng/mg Cr)
Normal	50	25/25	40–80 (mean: 65)	21.04 ± 1.98[c]
Transitional cell carcinoma	52	33/19	42–83 (mean: 64)	11.63 ± 1.29
Hematuria[a]	43	26/17	31–84 (mean: 64)	14.36 ± 2.26
Free of tumor[b]	29	12/17	29–79 (mean: 59)	13.34 ± 2.54

[a] Patients have hematuria of inflammatory etiologies
[b] Patients had a history of transitional cell carcinoma, but were free of recurrence at follow-up
[c] Standard error

no consensus concerning the site of production. By using polyclonal antibody, Poulsen [23] showed EGF immunoreactivity in proximal tubular cells only. Lau et al. [14], however, found immunostaining in all tubular cells and collecting ducts, with more intense reaction in proximal tubules. Recently, Salido [24] described very briefly the localization of kidney EGF in the thick ascending limbs of Henle and distal convoluted tubules.

The aim of this study was to investigate the origin of urinary EGF and any association of urinary EGF levels with urological diseases. Analysis comprises patients with active tumors, those in remission, and those with inflammatory diseases of non-neoplastic etiologies.

Materials and methods

Collection and preparation of samples

The appropriate method of sample collection was determined by comparing the EGF levels in morning urine and in overnight samples. A total of 22 healthy adults (11 men and 11 women) were assessed. EGF levels in morning urine (y) correlated well ($r=0.95$) with overnight samples (x) and an equation of $y=0.8778\times+1.8841$ was obtained. Morning urine was thus used as standard sample in the subsequent analysis. Reference values were established on the basis of 50 adults, and computed separately for men and women. Fresh morning urine was collected in February, 1992 in the National Cheng Kung University Hospital, Taiwan. Briefly, supernatant of urine was stored immediately after cytospin centrifugation at −70°C for EGF determination. Each sample was thawed and subjected to pH adjustment (pH: 7.4 ± 0.1) before assay.

The number of study cases and basic information are listed in Table 1. All fresh TCCs were documented by histopathology. Patients with hematuria of non-neoplastic etiologies ($n=43$) had a clinical diagnosis of cystitis ($n=24$), urinary lithiasis ($n=10$) and chronic pyelonephritis ($n=9$). The tumor-free group comprised patients who had undergone previous treatment for bladder cancer or renal pelvis carcinoma, but were still in remission at follow-up.

Assay procedure

EGF was measured within 2 months of storage by a homologous radioimmunoassay (Diagnostic Systems Laboratories, Tex.). All sheep anti-EGF antiserum, standards and EGF urine controls were provided by the manufacturer. The reconstitution and assay procedure followed the protocol recommended. Briefly, samples were incubated with ^{125}I EGF and EGF antiserum for 1 h at room temperature. Following precipitation all tubes were centrifuged, decanted, and drained. The radioactivity was counted in a multi-well gamma counter, and the results calculated using standard logarithmic curve fit. Each sample was assayed in duplicate. Recovery and cross-reactivity studies were conducted by spiked with recombinant human EGF and transforming growth factor-alpha (Oncogene Science, Cambridge, M.A.) in 0 ng/ml standard, up to 300 ng/ml respectively. The lowest detectable level of EGF is 1 ng/ml at the 95% confidence limit. The inter- and intra-assay coefficients of variation ($n=10$) were less than 10%. To correct for any water excretion effect, each aliquot of urine was assayed for creatinine (Synchron CX3 autoanalyzer, Beckman Instruments, Brea, Calif.). Relative urinary EGF concentration was expressed as a ratio of EGF to creatinine (ng/mg creatinine).

Immunohistochemistry

Paraffin blocks of non-neoplastic human kidney ($n=9$) obtained from laceration injury were selected to investigate the origin of EGF production. They were sectioned and submitted for deparaffinization. Monoclonal anti-human EGF antiserum (Santa Cruz Biotechnology, Santa Cruz, Calif.) was used as the primary antibody. Optimal dilution and staining conditions were determined using human salivary glands [23]. Sections were first washed for 5 min with phosphate buffered saline (PBS), and blocked with 0.1 mol/l HCl for 20 min. Then they were covered with 3% normal horse serum for 15 min. Primary antibody was diluted at 1:100 and incubated for 1 h at room temperature. The LSAB kit (DAKO corporation, Carpinteria, Calif.) was adopted as instructions for blocking, linkage, and labelling of staining procedure. 3.3′ Diamino-benzidine (DAB) was used as the chromogen. The sections were counter-stained with hematoxylin. Negative control was performed by incubation of non-immune mouse IgG in substitution for the primary antibody.

Statistical analysis

Simple linear regression was used for correlation analysis, and analysis of variance (ANOVA) was used for evaluation of the differences between the means. Only those variables with a P-value of less than 0.05 were considered significant.

Results

Table 1 demonstrates the mean EGF values in each group of urological diseases. There was a significant difference between the normal controls and each subgroup of patients ($P<0.005$), but, no apparent difference was observed among patients with active TCC, those in remission, and those with hematuria of non-neoplastic origin ($P>0.1$).

The impact of nephrectomy upon urinary EGF excretion is shown in Fig. 1. Patients with one kidney removed ($n=8$) had significantly lower mean values of EGF ($P<0.05$) than those with intact kidneys ($n=21$). There was no sex difference ($P>0.1$ respectively).

Tumor resection in relation to nephrectomy was analyzed to understand their effects upon urinary EGF (Fig. 2). Patients who had had one kidney removed for carcinoma ($n=8$) had significantly lower EGF values

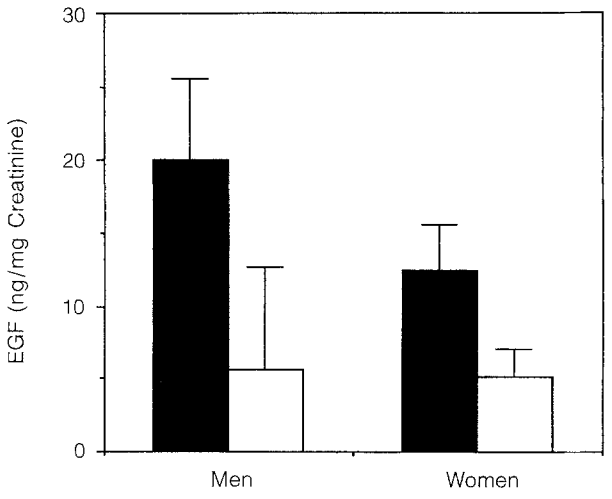


Fig. 1 Urine of patients who had undergone unilateral nephrectomy contained significantly lower levels of epidermal growth factor (EGF) than that of those who had intact kidneys, regardless of sex ($P < 0.05$). ■ Without nephrectomy; □ post-nephrectomy

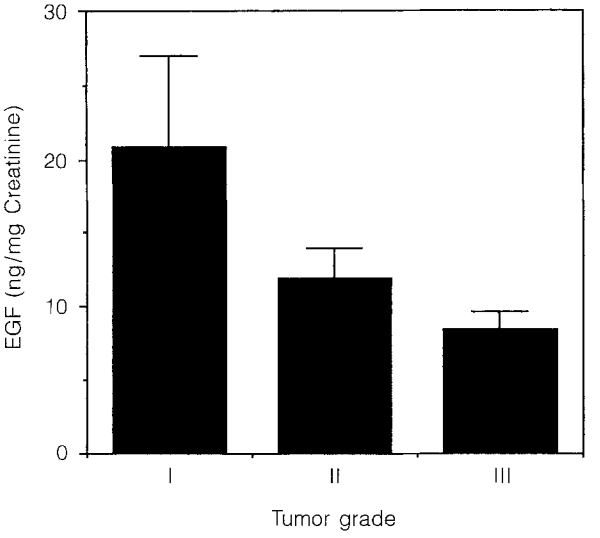


Fig. 3 The mean EGF urine levels declined with increasing tumor grade ($P < 0.02$) through grade 1 ($n = 7$), grade 2 ($n = 23$) and grade 3 ($n = 17$) carcinomas

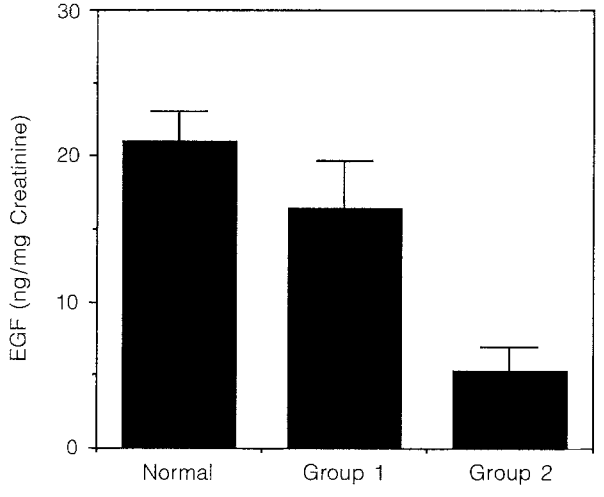


Fig. 2 The EGF concentrations of patients who had undergone bladder tumor resection but still had intact kidneys (group 1) were not statistically different from those in normal controls ($P = 0.2$). Patients who had had one kidney removed for pelvic carcinoma (group 2) had lower mean EGF values than normals ($P < 0.005$) or patients with intact kidneys ($P < 0.05$). The status with regard to nephrectomy is thus the most important factor in determination of EGF urine

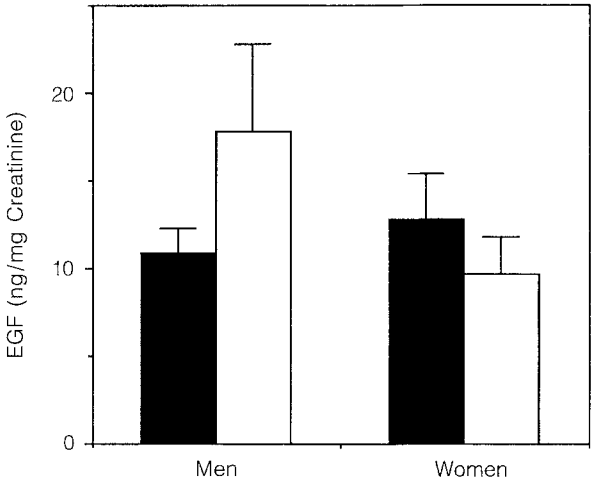
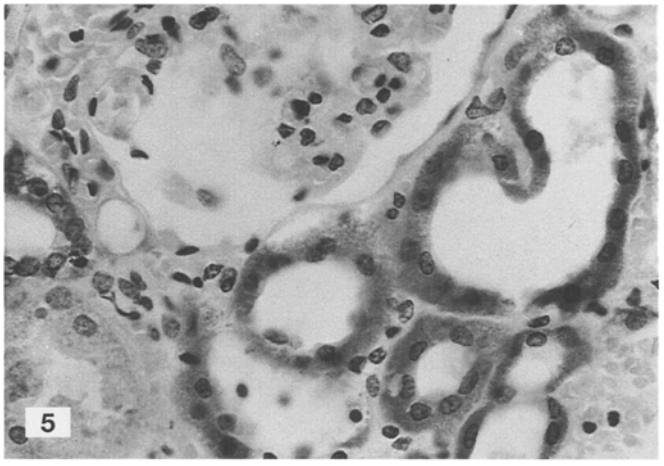


Fig. 4 Male patients in tumor-free status had significantly higher EGF urine levels than those with active tumors ($P = 0.03$), but no statistically significant difference was observed for female patients ($P = 0.91$). ■ Fresh tumor; □ tumor-free

($P = 0.04$) than patients with bladder tumor resection only ($n = 21$) and than normal controls ($P < 0.005$). In contrast, mean EGF urine levels of patients who had undergone bladder tumor resection were not statistically different from those in normal controls ($P = 0.2$). Altogether, nephrectomy is the most important factor in detecting urinary EGF.

The association of EGF urine levels with TCC was evaluated in relation to tumor grade. There was a significant decline of urinary EGF values with increasing tumor grade ($P < 0.01$). Five patients were excluded because unilateral nephrectomies had been performed for grade 2 pelvic carcinoma. The reverse relationship between EGF levels and tumor grade was statistically significant ($P < 0.02$) as shown in Fig. 3.

The difference in mean urinary EGF values between the patients with active TCC ($n = 52$) and those in remission ($n = 29$) were not statistically significant ($P = 0.5$) in either men or women ($p > 0.1$ for both sexes). Figure 4 demonstrates the results after exclusion of the patients



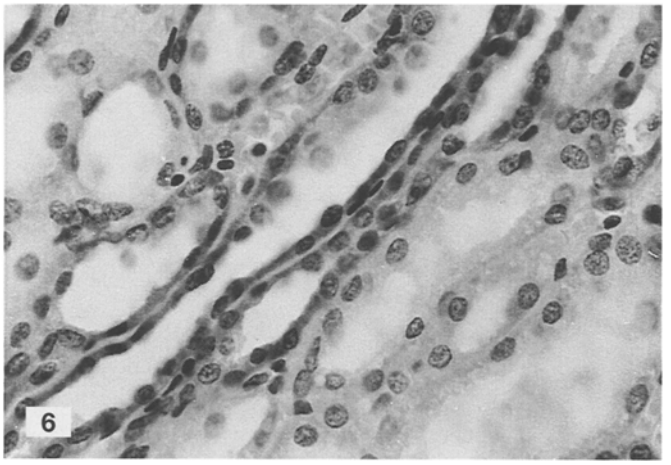


Fig. 5 Immunohistochemical staining of epidermal growth factor revealed strong reactivity in the distal tubular cells of non-neoplastic kidneys, whereas the proximal tubular cells were non-reactive. The staining was essentially in diffuse pattern. ×500

Fig. 6 The thick ascending limbs of Henle showed an intense and diffuse staining reaction, whereas the collecting tubules were not reactive for epidermal growth factor antibody. ×500

who had undergone nephrectomy. Men with a history of TCC had significantly higher EGF urine levels than those with active TCC ($P=0.03$), but this was not a statistically significant factor in women patients ($P=0.91$).

The distribution of EGF in human kidneys was studied by immunohistochemistry. There was diffuse and homogeneous staining in the distal convoluted tubules (Fig. 5) and thick ascending limbs of Henle (Fig. 6). The proximal tubules showed occasional weak staining in the basal portion. Both collecting ducts and glomeruli were essentially negative. There was no reaction in the interstitium of the renal cortex, medulla and urothelium.

Discussion

Early experimental studies have demonstrated the important role of EGF in normal urothelial turnover and its ability to enhance tumor growth [11, 18]. Based on the correlation of EGFR with tumor behavior, it is suggested that EGF has critical effects on bladder cancer [1, 20, 21]. The association of low EGF values with urothelial carcinomas and recovery after tumor resection in the current study, strongly support the significance of EGF being involved in tumor behavior, as has previously been suggested [1, 5, 10, 20, 21]. However, the decline of EGF urine levels with increasing tumor grade is in contrast to data presented by Fuse et al. [5]. In terms of the correlation of tumor grade to EGFR density, our data thus confirm the importance of EGF/EGFR interaction in the biology of urothelial carcinomas [20, 21].

However, the mechanism needs to be mediated by cellular expression of EGFR. Receptor status examined by immunohistochemical method revealed that about half, ranging from 48% to 82%, of the urothelial carcinomas belong to this category [3, 18, 21]. The expression of EGFR is significantly associated with tumor proliferation, as defined by Ki-67 index [3]. Furthermore, it is relevant in terms of recurrence rate, time to recurrence, and death from cancer [21]. Hence, it would be necessary to compare the EGF values in relation to the receptor status of tumors and follow its response to treatment longitudinally. The usefulness of monitoring its changes in predicting recurrence of EGFR-positive tumors is being undertaken and will be reported on later.

We found that patients with hematuria of non-neoplastic diseases of the urinary tract had lower mean EGF levels than age-adjusted controls. This is different from earlier reports on chronic cystitis patients [8]. Expression of EGFR does not appear on the superficial layer of urothelium in urinary tract inflammation [18]. In this regard, mucosal erosion in cystitis or urolithiasis will expose EGFR of intermediate and basal cells of the urothelium and favor its interaction with urinary EGF. Thus, it seems likely that a low EGF urine level is consumed and involved in the processes of tissue repair. We demonstrated that the measurement of urinary EGF per se could not be an effective tumor marker, which is rational considering its wide range of responses in pathophysiology. The change in EGF values may imply either reaction to mucosal injury or possible involvement in tumor biology, but only men had significant elevation of EGF values after tumor resection. Currently, we have no idea of the sex differences in EGF conversion. This is opposed to data presented in previous report [5]. On exclusion of associated infection, the only feasible explanation at this time seems to be a continuous consumption of EGF within the urinary tract of women. In terms of EGF/EGFR interaction the concept of "field change" should be considered. It means abnormal expression of EGFR on grossly normal urothelium adjacent to, or remote from, primary tumors [18]. The abnormal distribution and residual expression of EGFR may provide fertile soil through which new recurrence could be promoted. Our data seems to support the hypothesis of field change for women.

Yet there was no information dealing with the sex difference in relation to EGFR expression [7, 18]. In our previous study, the EGFR status showed no correlation ($P > 0.5$) with the sex of the patients [3]. In addition, the sex variable did not have any prognostic significance in relation to patient survival [21, 22], tumor recurrence [9] or progression for superficial bladder cancer [7] or low-grade papillary carcinoma [6]. In this context, further investigation on a larger scale is needed to verify the relevance of our observation.

When we compared the impact of nephrectomy on EGF levels, our findings supported the idea that most urinary EGF comes from the kidney, as had previously been suggested [4, 11, 14]. In the early post-surgical period, Coutre [4] showed a drop in EGF by less than 50% of the expected range. A possible functioning of EGF in compensatory, hypertrophic renal tissue was suggested. This hypothesis was supported by demonstration of increased distal nephron EGF content and altered distribution in residual hypertrophic rat kidneys [19]. Our examples were analyzed at 6 months to 9 years after nephrectomy. Since there was no additional reduction in EGF urine levels in these patients, it is suggested that EGF is secreted on the basis of renal mass and therefore does not recover its preoperative range. As far as the site of production is concerned, we found an immunostaining reaction in the distal convoluted tubules and thick ascending limb of Henle. This is essentially similar to results published by Salido [24] and Lakshmanan [13], but different from those obtained using polyclonal antibody [14, 23].

In summary, we support the renal origin of most urinary EGF excretion. Our results indicate a decrease of epidermal growth factor in inflammatory diseases of the urinary tract. Furthermore, EGF may play an important role in the biological activity of TCC. A longitudinal study is required to clarify its value as a marker of tumor recurrence for receptor-positive urothelial carcinoma.

Acknowledgements This work is supported by grant NSC-81-0412-B-006-545 from the National Science Council, ROC. We are grateful to Professor Robert E. Hurst (Department of Urology, Health Science Center, The University of Okalahoma) for his critical review of the manuscript and valuable suggestions.

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