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Epidermal growth factor in human breast cancer, endometrial carcinoma and lung cancer. Its relationship to epidermal growth factor receptor, estradiol receptor and tumor TNM

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Summary

Epidermal growth factor (EGF) and its receptor (EGFR) were measured in 60 breast cancers (BC), 6 benign mammary tumors (BM), 8 samples of normal breast (NB), 6 endometrial carcinomas (EC) and 30 lung cancers (LC). EGF was measured in plasma, saliva and urine from 20 patients with BC, before and after tumor excision, and in 8 patients with metastatic disease. The median EGF in BM and BC was significantly higher ($P < 0.05$) than in NB. No significant correlation between EGF and EGFR was found in BC. Neither tumor excision nor the spreading of the disease significantly modified the EGF concentrations in biological fluids. In LC there was an inverse relationship between EGF and EGFR ($rs = -0.36$; $P = 0.09$), which disappeared in normal lung. It is concluded that EGF may play a role in malignant transformation; however, the weak correlation between EGF and EGFR lessens the importance of EGF in either autocrine or paracrine stimulation of tumor growth.

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Introduction

The stimulatory effect of EGF on several human tumor cell lines [1] supports the hypothesis that EGF, by its binding to its membrane receptor (EGFR), may promote tumor growth in an autocrine or paracrine way [2–4]. The tumor growth factor α (TGFα), due to its structural similarity with EGF, shares the same membrane receptor [2–4]. EGF has been found in human urine [5–7] as well as in saliva, plasma and milk [4] from normal human individuals. Several studies had shown that urinary EGF excretion is higher in women with breast cancer (BC) than in matched controls [8] and that excretion of high molecular mass forms of TGFα were also raised in patients with BC [9–13].

Although the urinary excretion of EGF and TGFα in patients with BC has been reported [10–12], little is known about the source of these polypeptide growth factors. Cultured tumor cell lines may secrete polypeptide growth factors [15–18], and a few recent studies had demonstrated a higher production in cells from BC tumors [19,20]. Although there are some studies on TGFα in tumor tissues little is known about the simultaneous presence of EGF and EGFR in normal tissues and/or human malignancies [20]. All of the above has prompted us to assess the importance of EGF in human BC, endometrial carcinoma (EC) and lung cancer (LC) and to evaluate the possible relationships between EGF, EGFR and estradiol receptors (ER) in BC and EC.

Material and Methods

Subjects

The following human tissues were included: 60 BC specimens from patients with a median age of 53 years (51 of the tumors were ductal carcinomas, 3 medullary, 2 lobular and 4 belonged to other histopathological types); 6 benign mammary tumors (BM) (3 phyloids, 2 fibroadenomas and 1 mammary cyst); 7 specimens of excised surrounding normal breast (NB) and 1 case of gynaecomastia; 6 EC from patients with a median age of 60 years and 30 LC from patients with a median age of 62.5 years (19 being epidermoid carcinomas, 7 bronchial adenocarcinomas and 4 belonged to other types). In most of these cases a sample of the surrounding normal lung was also studied.

EGF, EGFR and ER were measured in BM, BC and EC; in LC and normal lung, EGF and EGFR were determined.

The tissue samples were brought from the theatre to the laboratory in transport buffer (10 mM HEPES, 0.15 M NaCl, pH 7.4) in less than 30 min. In the laboratory they were cleaned of fat, blood or necrotic tissues and the clean tumors were stored in liquid nitrogen until the assay was carried out.

EGF in biological fluids

EGF was measured in plasma, saliva and urine from 8 patients with BC with one or more metastatic foci in lymph nodes, bone and/or viscera. To assess a possible effect of tumor excision on EGF, plasma, saliva and urine, EGF was determined in the biological fluids from 20 BC patients, before and two weeks after tumor excision.

EGF in biological fluids was also assessed in age matched reference groups consisting of normal women free from malignancies. The biological fluids were stored at -20°C until the assay of EGF was carried out.

Methods

EGF and EGFR determination

Reagents. Mouse EGF receptor grade (mEGF) and the recombinant human EGF (hEGF) were from Biomedical Technologies Inc. [^{125}I]mEGF (164–178 μCi/μg, New England) and [^{125}I]hEGF (>6000 Ci/mmol, Amersham) were used as labels ligands. Rabbit anti-γ-globulin was used as the second antibody (Milles-Yeda).

EGF tissue extraction. All of the steps in the homogenization were performed in an ice bath. Tissue EGF was extracted following acetic acid extraction [21]. Between 150 and 300 mg of tissue were homogenized in 2 ml of 85 mM acetic acid. The homogenate obtained was centrifuged at 500–1,000 × *g* for 5 min at 4°C, and the supernatant, containing the EGF, was stored at -20°C until assay.

Determination of EGF. The EGF extracts were adjusted to pH 7.0 by the addition of 0.5 M NaOH using pH indicator strips. Plasma, saliva and urine were diluted with distilled water in test tubes.

EGF was determined using the heterogeneous radioimmunoassay of Starkey and Orth [6] with our own antibody raised in rabbit against mouse EGF (mEGF, Biomedical Technologies Inc). The antibody showed cross-reactivity with human EGF (hEGF) in the assay conditions using [^{125}I]hEGF and hEGF in the standard curve. The specificity of the antibody used was studied by checking its cross-reactivity with TGFα and the following polypeptide hormones: ACTH, FSH, HCG, TRH, GH, insulin and glucagon, at a concentration 100 times that of hEGF of the standard curve. 0.1% cross-reactivity for HCG was detected and was negligible or almost nil for all the other compounds tested.

The standard curve used for measuring hEGF was prepared with recombinant hEGF with seven standards ranging in concentration from 0.07 to 5 ng/ml. Bound and free fractions were separated using a rabbit anti-γ-globulin (Milles Yeda). Recombinant [^{125}I]hEGF (Amersham) was used as labelled ligand.

To increase sensitivity the assay was performed using two incubation steps. During the first step, the EGF of samples, and of the standards, were incubated with the first antibody for 12 h at 4°C, then 100 μl [^{125}I]hEGF were added and the incubation continued for 36 h at 4°C. Finally, rabbit anti-γ-globulin plus rabbit serum were added, incubated for 12 h at 4°C and centrifuged at 4,000 × *g* to separate the fractions.

The standard curves were fitted to the Logit-Log model. The between assay cv. was 16% for values of EGF <0.1 ng/ml and 12% for higher values. The linearity was demonstrated in urine samples from values of 0.2–4 ng EGF/ml. The concentrations found in the mammary tissue have <0.2 ng EGF/ml. The final results of tissue samples were expressed as ng EGF/mg of soluble total protein (TP), determined by the Bradford method [22]. The results in plasma and saliva were expressed in ng EGF/ml and as ng EGF/mg creatinine for urinary samples.

Sample preparation for EGFR and ER. The same sample homogenate was used for ER and EGFR determination. A minimum of 150 mg of tissue was homogenized in

HEPES-EDTA dithiothreitol buffer (20 mM HEPES, 1.5 mM EDTA, 0.125 mM dithiothreitol, pH 7.4), 1 ml for each 50 mg tissue. After the tissue was homogenized, it was centrifuged at 5,000 × *g* for 15 min at 4°C to yield a cytosolic supernatant and a nuclear pellet. The nuclear pellet was kept for nuclear ER (nER), but the supernatant was centrifuged at 25,000 × *g* for 30 min at 4°C. The supernatant was kept for cytosolic ER (cER) and the sedimented plasma membranes were resuspended in 1/2 of the original volume with assay buffer (1 mM HEPES, 5 mM NaCl, pH 7.4, containing 1 g BSA/l). A small portion of cytosolic fraction was centrifuged apart to determine plasma membranes TP using the Bradford method [22].

EGFR determination. A two-point saturation assay on duplicate samples [23] was used, consisting of two tubes containing 100 μl of plasma membranes plus 100 μl [^{125}I]mEGF (50,000 cmp ≈ 300–400 fmol/ml) and 200 μl of assay buffer to evaluate the total binding; also, two tubes in which the 200 μl of assay buffer were substituted by 200 μl of a solution containing 8333 fmol of non-radioactive EGF, to evaluate the non-specific binding. The tubes were incubated for 16 h at 4°C and then centrifuged at 20,000 × *g* for 10 min at 4°C. Specific binding was obtained by subtracting the mean counts/min of non-specific binding from that of total binding. Results were expressed in terms of fmol ^{125}I-EGF bound to plasma membranes/mg plasma membrane TP.

Homogenates of placental tissue (rich in EGFR) were used in each assay as positive controls. A tumor was considered positive (EGFR+) when EGFR concentration was >0.5 fmol/ml homogenate being the non-specific binding <70% of total binding[23].

ER determination. ER was determined following Leake's method [24]. Fifty microliters of [^{3}H]-E_2 with or without 100 times diethylestilbestrol in 7 increasing concentrations in the 10^{-10}–10^{-9} M range were added to 150 μl of cytosolic and nuclear fractions. All the tubes were incubated for 18 h at 4°C. The [^{3}H]-E_2 receptor complex was separated from the free [^{3}H]-E_2 using the dextran coated charcoal method for the cytosolic fraction and by filtering for the nuclear fraction.

The results were expressed in fmol cER/mg of TP for the cytoplasmic fraction and fmol nER/mg DNA for the nuclear pellet. The DNA was determined following the Katzellenbogen modification of Burton's method [25].

A tumor was considered ER positive (ER+) when ER was simultaneously present in the cytosolic (cER+) and nuclear fractions (nER+), and ER negative (ER–) when it was not present in either cellular fraction (–/–) or was present in only one of them (+/– or –/+).

Statistics

Median and ranks were used instead of means and standard deviations because non parametric statistics were applied. To compare the results of two or more groups the Mann-Whitney (MW) or Kruskal-Wallis (KW) tests were used. To study the correlations between EGF and EGFR the Spearman rank correlation coefficient (*rs*) was used [26].

Results

The EGF content in BC did not show any significant statistical variation in relation to tumor size, node involvement or EGFR status (Table I). However, the

presence of metastases was associated with a significantly lower median EGF content in comparison with those without metastases (KW = 4.45; $P < 0.05$; Table I). ER shows a similar trend, although the difference in medians did not reach the levels of statistical significance (Table I).

EGF tended to increase parallel to the BC EGFR content, although the correlation coefficient did not reach the limits of the statistical significance ($rs = 0.31$; $P = 0.17$). The median EGF content of NB was significantly lower (KW = 6.78; $P < 0.05$) than the EGF concentration found in benign mammary tumors or in BC, both of which have similar medians (Fig. 1).

The plasma, saliva and urine concentrations of EGF in patients with BC did not show any significant variation after tumor excision (Fig. 2A), although, contrary to

TABLE I

EGF in human breast cancer

Parameter	*N*	Median (ng EGF/mg TP)	Range (ng EGF/mg TP)
Menstrual status			
Premeno	21	0.19	0.00–0.87
Postmeno	39	0.12	0.01–1.11
KW (*P*)		1.88 (0.17)	
T			
T1	7	0.11	0.00–0.19
T2	28	0.14	0.03–0.87
T3	10	0.19	0.03–0.36
T4	13	0.11	0.01–1.11
KW (*P*)		1.84 (0.64)	
N			
N = 0	24	0.12	0.00–0.55
N = +	35	0.19	0.01–1.11
KW (*P*)		1.56 (0.21)	
M			
M = 0	46	0.18	0.01–1.11
M = 1	4	0.07	0.01–0.11
KW (*P*)		4.45 (0.034)	
ER			
ER+	18	0.09	0.00–1.11
ER-	42	0.18	0.01–0.87
KW (*P*)		3.03 (0.08)	
EGFR			
EGFR+	20	0.16	0.00–0.50
EGFR-	39	0.13	0.01–1.11
KW (*P*)		0.08 (0.85)	

KW, Kruskal-Wallis test; *N*, number of cases; *P*, significance level.

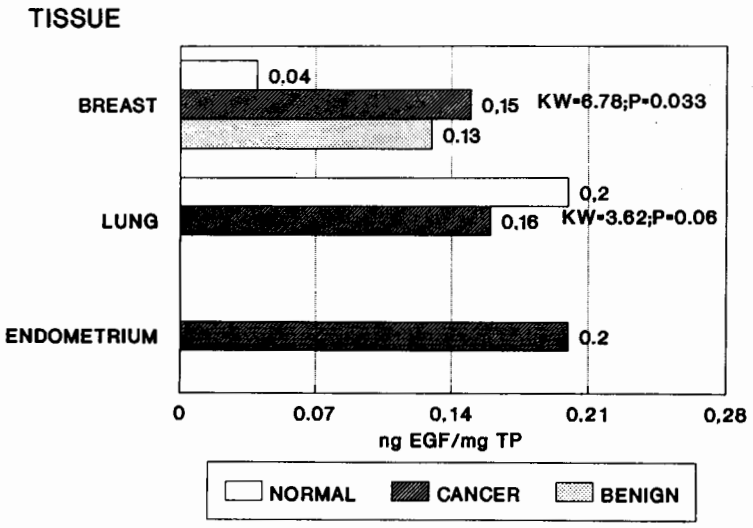

Fig. 1. Median EGF concentrations in different human tissues and malignancies.

what is expected, the urinary excretion of patients with cancer is lower than the one of reference group (Fig. 2A). The presence of distant metastases did not seem to modify significantly the EGF concentration in the body fluids with regard to the reference group (Fig. 2B), although the patients with metastases excrete higher concentrations of EGF than the reference group (Fig. 2B).

Five of the 6 EC were EGFR+ and all were ER+ (Table II). Their median EGF content, 0.2 ng/mg TP (Fig. 1), was very similar to the EGF content in normal lung. No association was found between EGF content and tumor TNM or EGFR status in LC (Table III). The median EGF in normal lung was higher than that in lung cancer, although the difference was not statistically significant (Fig. 1). A negative trend between EGF and EGFR was found in lung cancer (Fig. 3B) but disappeared in normal lung (Fig. 3A).

Discussion

The presence of higher levels of EGF in benign mammary tumors and BC when compared to NB supports the suggestion that EGF might have a role in the process of breast malignant transformation [27–29]. They are in accordance with the high levels of EGF found in the liquid of mammary cysts [28,29] and correspond with the higher significant levels of TGFα or even gene amplification found in some benign tumors [19]. The slight correlation found between EGF and EGFR might be attributable to the internalization of EGFR due to its binding with either EGF or TGFα. On the other hand, the low importance of EGF in breast tumors is reinforced

by the negligible amounts of EGF found in other studies [20]. Therefore, if EGFR is to be stimulated as a part of the transformation/progression pathway, it is more likely to be TGFα than EGF which might play the major role.

While the lower levels of EGF found in BC with metastasis are statistically significant, the scarcity of cases lessens the importance of this finding.

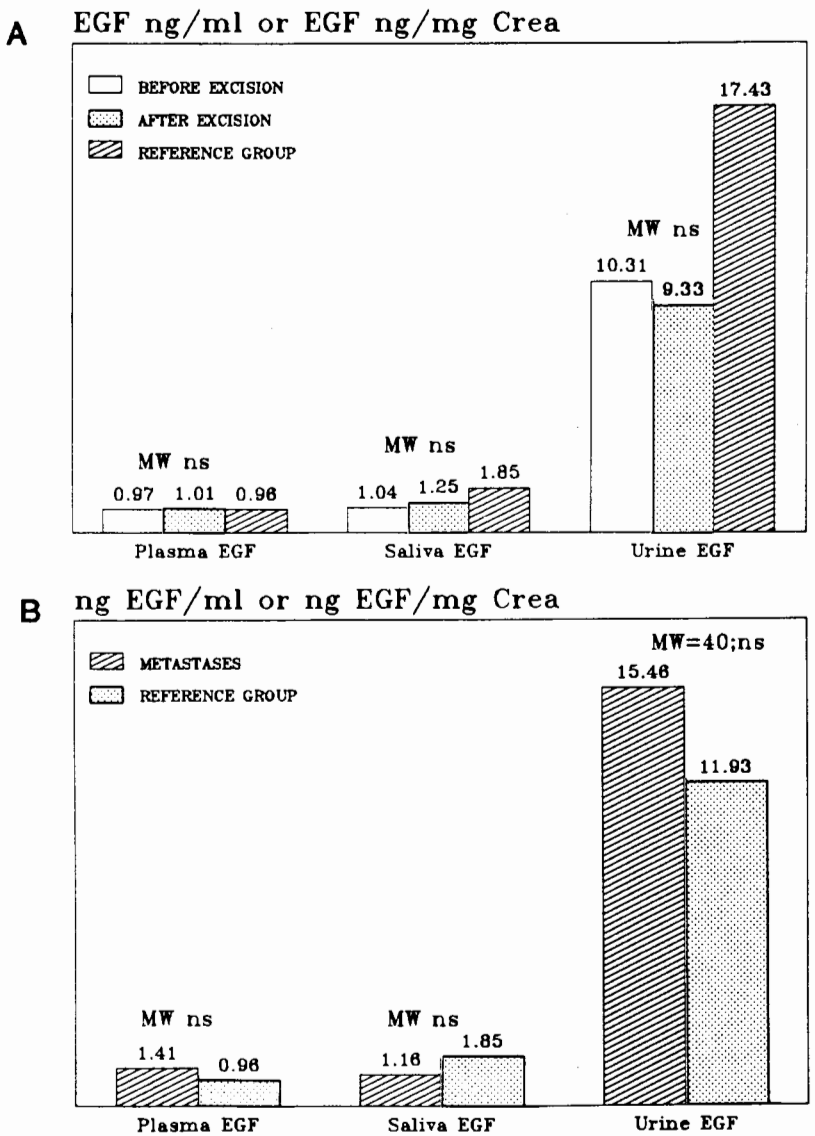

Fig. 2. (A) Median EGF levels in plasma, saliva and urine before and after BC excision compared to reference group. (B) Median EGF concentrations in the biological fluids of patients with BC in the stage of spreading metastasic disease with regard to reference group.

TABLE II

EGF in endometrial carcinoma

Parameter	*N*	Median (ng EGF/mg TP)	Range (ng EGF/mg TP)
ECFR			
EGFR+	5	0.22	0.16–0.30
EGFR–	1	0.17	0.17–0.17
KW (*P*)		0.08 (0.76)	
ER			
ER+	6	0.22	0.16–0.30
ER–	0		

N, number of cases; *P*, significance level.

The results of the present study do not indicate the ability of BC cells to secret enough amounts of EGF as to modify the EGF levels in the biological fluids, which is in congruence with other study in this field in which the patients with BC showed a significantly low urinary output of EGF with regard to their matched controls [20]. Other studies carried out in biological fluids of patients having several kinds of

TABLE III

EGF in lung cancer

Parameter	*N*	Median (ng EGF/mg TP)	Range (ng EGF/mg TP)
T			
T1	7	0.15	0.04–0.28
T2	16	0.16	0.02–0.35
T3	5	0.15	0.00–0.40
KW (*P*)		0.37 (0.83)	
N			
N = 0	13	0.16	0.05–0.59
N = +	16	0.16	0.00–0.35
KW (*P*)		0.46 (0.49)	
M			
M = 0	24	0.16	0.00–0.40
M = 1	1	0.02	0.02–0.02
KW (*P*)		2.32 (0.13)	
EGFR			
EGFR+	24	0.15	0.00–0.59
EGFR–	6	0.19	0.05–0.28
KW (*P*)		0.21 (0.64)	

N, number of cases; *P*, significance level.

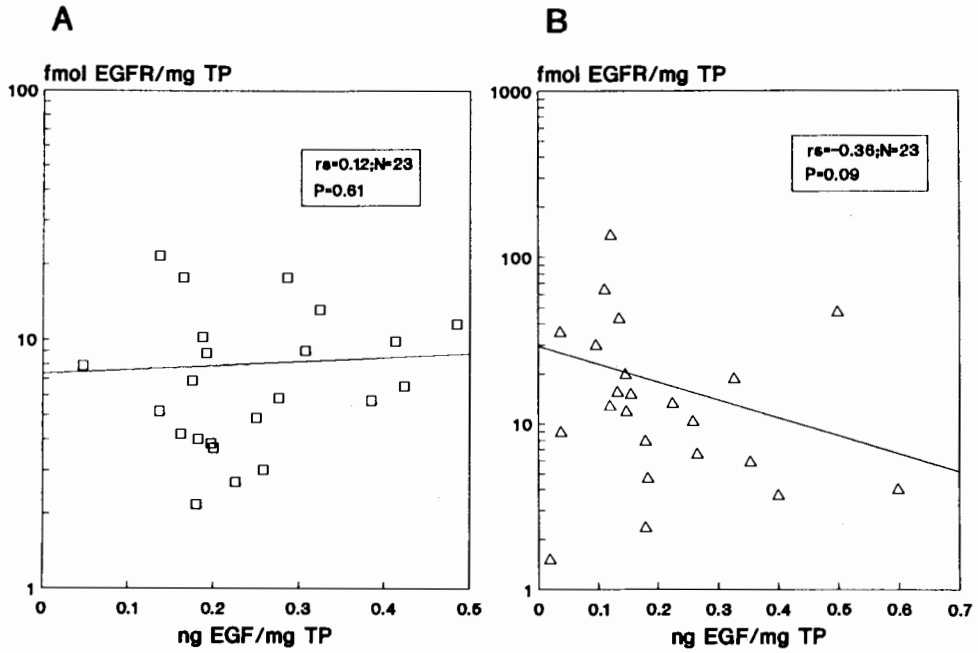

Fig. 3. Relationship between EGF and EGFR in normal lung (A) and in LC (B). *rs*, Spearman rank correlation coefficient.

malignancies also failed to find significant differences [30]. However, urinary excretion of higher molecular forms of EGF or TGFα has been found in patients with cancer [11–14].

These results suggests that the presence or absence of tumor is irrelevant, since the levels of EGF not only did not vary after tumor excision, but on the contrary, the BC patients excreted lower levels of EGF than the reference group. The presence of metastasis does not suffice to significantly modify the EGF, although the patients with metastases had a higher urinary excretion of EGF than the reference group. The variations in biological levels of EGF may be irrelevant or they may lend support to a host-reaction hypothesis rather than a tumor production mechanism.

In lung tissue, and contrary to the findings in breast tissue, malignant transformation is associated with a decrease in EGF content. Moreover, a slight negative trend between EGF and EGFR appears in lung cancer, but is almost non-existent in normal lung. These findings suggested that EGF and EGFR might play a different physiological role in lung tissue when compared to breast tissue.

From the above data we can conclude that, although EGF might play a role in the process of malignant transformation in BC, there is no evidence that attributes EGF in previously established BC. The weak correlation between EGF and EGFR lessens the importance of EGF in favor of TGFα, in the autocrine or paracrine way of tumor growth. The different patterns of EGF and EGFR in lung and breast suggested that EGF and EGFR might have different physiological significance in each tissue.

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