Characterization and Quantitation of the Epidermal Growth Factor Receptor in Invasive and Superficial Bladder Tumors

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ABSTRACT

Epidermal growth factor receptors (EGF-Rs) have been measured on primary human bladder tumor membranes by 125I-EGF ligand binding. High affinity receptors were detected on both superficial (Kd, 0.2-1.45 nM; mean, 0.86 nM; median, 0.88 nM) and invasive tumors (Kd, 0.19-2.38 nM; mean, 0.9 nM, median, 0.79 nM). There was one class of binding sites and EGF receptor concentration was quantified by competitive binding and Scatchard analysis. The EGF receptor was further characterized and shown to be cleaved at the major autophosphorylation site by a calcium-activated mechanism. Thus the EGF receptor from primary bladder tumors exhibits similar biochemical characteristics to those in established cell lines. Tumors classified as invasive on the basis of muscle invasion had higher EGF levels [EGF binding, 99 ± 252 (SD) fmol/mg protein; median, 21; n = 24] than superficial tumors (12 ± 12 fmol/mg protein; median, 11; n = 23) or normal bladder mucosa (9 ± 12 fmol/mg protein; median, 6; n = 6) (P = 0.05). When the two largest subgroups of superficial and invasive tumors were compared (15 pT2, 16 T3), the invasive tumors had significantly higher EGF levels (P < 0.05). EGF may therefore be involved in mechanisms of tumor progression. EGF-R may be a target for selective therapy with EGF-linked drugs in a subset of invasive bladder cancers.

INTRODUCTION

EGF is a 53-amino acid peptide with a molecular weight of approximately 6000 (1) which contains 3 disulfide linkages. Originally isolated by Cohen from the submaxillary gland of the male mouse (2), it was identified independently in human urine by Gregory (3) and has been found to be a potent stimulator of cell growth and division in cells of many different types. TGF-α is secreted by many transformed cells and is also present in various tumors and normal tissues. It shows considerable sequence homology to EGF. Secretion of TGF-α may be an important mechanism for autocrine growth stimulation.

The actions of EGF and TGF-α are mediated by binding to a specific membrane-bound receptor (EGF-R) which has a close structural relationship with the oncogene product of erb-B of the avian erythroblastosis virus (4), gp65<sup>5/58</sup>-8, which represents a truncated EGF-R which lacks the external binding site for EGF. The intracellular portion of the EGF-R has an associated tyrosine kinase domain and three tyrosine residues which are autophosphorylated after binding of EGF to the receptor. Several other growth factor receptors and many oncogene proteins also have tyrosine kinase activity.

EGF-R have now been identified on cells from several common human tumors or tumor cell lines. The receptors have been identified by several methods including immunohistochemical staining for the receptor, ligand binding, or analysis of DNA or RNA. Increased levels of receptor have been found in breast cancer (5, 6), gliomas (7), lung cancer (8), bladder cancer (9-11), and tumors of the female genital tract (12, 13). Amplification of the EGF-R gene has also been identified sporadically in cell lines including the A431 cell line which was originally a vulval squamous carcinoma (14), and in such tumors as gliomas (7) and bladder tumors (11). However, the frequency with which gene amplification leads to increased EGF-R expression varies with tumor type. Thus in human bladder cancers it was found in only 1 of 29 tumors studied (11), whereas in gliomas the incidence was much higher (7). Whichever mechanism of regulation occurs it is ultimately the expression of functional EGF-R that mediates the effects of TGF-α.

Analysis of the EGF-R content by means of immunohistochemistry has suggested that EGF-R positivity may be associated with features of poor prognosis such as high tumor stage, high grade, and absent estrogen receptors (6, 10). However, histochemistry is difficult to quantify and it does not characterize the receptor in terms of affinity and molecular weight or function, both of which are relevant data for potential drug targeting. In view of this, we decided to study further the EGF-R in human bladder cancer. By means of ligand binding we have quantified the expression of the EGF-R and have determined that the receptor is functional in that it autophosphorylates after addition of EGF. We have shown quantitative differences in the numbers of binding sites between superficial and some invasive tumors.

MATERIALS AND METHODS

Tissue Samples. We studied 47 patients with bladder cancer; 35 were male and 12 female. The mean age was 68 ± 9 (SD) years (range, 45-86 years). The Union International contre le Cancer system of tumors-metastasis classification was used to stage the tumors (Table 1).

Twenty-three patients had tumors that did not invade the bladder muscle (15 pT2, 8 pT1). One was well differentiated, 17 were moderately differentiated, and 5 were poorly differentiated. Twenty-four patients had tumors that invaded bladder muscle (4 T2, 16 T3, and 4 T4). 7 were moderately differentiated tumors, and 17 were poorly differentiated.

Bladder samples were obtained from 6 control patients (mean age, 59 ± 17 years) who did not have bladder cancer. Four men were undergoing prostatectomy for bladder outflow obstruction and 2 women were undergoing bladder augmentation. Samples were obtained by means of a resectoscope using the diathermy cutting current. The samples were placed in ice-cold saline before being snap-frozen in isopentane cooled to −150°C with liquid nitrogen. The delay before insertion in the isopentane averaged 30 min but was never more than 1 h. The samples were wrapped in aluminum foil to prevent dehydration and stored in liquid nitrogen until required.

Materials. Receptor grade EGF and iodogen were obtained from Sigma (Poole, Dorset, England). Disuccinimidyl suberate was from Pierce and Warriner (Chester, England). Sodium [125I]iodide and [γ-32P]ATP (specific activity, 3000 Ci/mmol) were from Amersham International Plc. (Amersham, Buckinghamshire, England).

Membrane Preparation. All procedures were carried out at 0-4°C. Tissues were thawed, minced finely, and homogenized in a ratio of 1:10 (w/v) in buffer A (0.01 M Tris-HCl 0.05 mM NaCl-0.1% bovine serum albumin, pH 7.4, at 20°C) using four 10-s bursts of a Polytron homogenizer at speed setting 7. The contents were continually immersed in

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2 The abbreviations used are: EGF, epidermal growth factor; TGF-α, transforming growth factor α; EGF-R, epidermal growth factor receptor; SDS, sodium dodecyl sulfate.
an ice-water bath and 60-s cooling periods were used between bursts. The tissue was further disrupted using a glass-glass homogenizer with a tightly fitting pestle. In some experiments, the minced tissue was pulverized in a porcelain mortar chilled in liquid nitrogen and the fine powder was homogenized in a glass-glass homogenizer. The homogenate was centrifuged at 100,000 x g for 40 min. The supernatant was centrifuged at 100,000 x g for 40 min and the pellet (crude membrane fraction) was resuspended in buffer A and stored at −70°C until assayed.

Protein concentrations were assayed using the method of Bradford (15), with bovine serum albumin as standard.

EGF Receptor Assay. EGF was iodinated by the iodogen method (16). Separation of 125I-EGF from unbound 125I was by passage through a column (2.2 × 30 cm; flow rate, 24 ml/h) of Sephadex G-50 fine. Specific activities of the 125I-EGF varied between 70 and 100 ßCi/µg. The binding assays were performed in triplicate in Eppendorf tubes in a final volume of 400 µl buffer A containing 125I-EGF (final concentration, 0.3 nM), 100 µg membrane protein, and increasing amounts of unlabeled EGF (to give 15 final concentrations between 0 and 100 nM). After 2 h at 26°C the reaction was stopped by adding 1 ml ice-cold buffer A and centrifuging at 14,000 × g for 8 min. The pellet was washed with 1 ml ice-cold buffer A and centrifuged again at 14,000 × g for 8 min. The tips of the Eppendorf tubes were cut off and counted in a LKB-Wallac 1272 Clinigamma γ-Spectrometer (LKB-Wallac, Turku, Finland) with a counting efficiency of 81%. Where insufficient tissue was present to carry out full Scatchard analysis, membranes were incubated with 125I-EGF (final concentration, 5 nM) in the presence and absence of unlabeled EGF (final concentration, 500 nM) and the difference between the two was taken as a measure of specific binding.

Affinity Labeling of EGF Receptor. Membrane preparations were incubated for 2 h at 26°C in a final volume of 100 µl buffer A containing 125I-EGF (final concentration, 7.5 nM) in the presence and absence of unlabeled EGF (final concentration, 750 nM). The membranes were washed with 1 ml ice-cold buffer A and centrifuged at 14,000 × g for 8 min. The membrane pellets were resuspended in 100 µl 1 mM disuccinimidyl suberate in 15% dimethyl sulfoxide and cross-linking of the solubilized receptor preparations: unlabeled EGF to a concentration of 1 µg/ml; calcium to 2 mM; and ethylene glycol bis(β-aminoethyl ether)-Ν,N′,Ν′,Ν'-tetraacetic acid to 2 mM. The incubation was continued for 8 min and the following additions were made to aliquots of the solubilized receptor preparations: unlabeled EGF to a concentration of 1 µg/ml; calcium to 2 mM; and ethylene glycol bis(β-aminoethyl ether)-Ν,N′,Ν′,Ν'-tetraacetic acid to 2 mM. The incubation was continued for 8 min. The reaction contents were cooled to 0°C and the samples were made 53 µM with respect to sodium o-vanadate, 1.1 µM with respect to MnCl₂, and 10.6 µg/ml with respect to aprotinin. Phosphorylation was started by adding 4 µCi [γ-32P]ATP (final concentration, 26.1 nM) and continued for 10 min at 0°C. The reaction was stopped by the addition of 0.5 volume of SDS sample buffer and heating to 95°C for 3 min prior to electrophoresis.

SDS Polyacrylamide Gel Electrophoresis. Electrophoresis was performed using an 8% running gel and a 4% stacking gel (17). Molecular weight markers were run on each gel. After electrophoresis (40 mA/gel for 5 h) gels were stained with 0.1% Coomassie brilliant blue in 10% acetic acid-10% propan-2-ol, destained in acetic acid-propan-2-ol, dried down, and exposed to Kodak X-omat X-ray film (Eastman-Kodak, Rochester, NY).

Membrane Marker Enzyme Assays. Alkaline phosphodiesterase I and 5′-nucleotidase were both assayed according to the method of Aronson and Touster (18).

**RESULTS**

Membrane Enzyme Markers. When comparing the activity of two membrane-associated marker enzymes in the various fractions there was a 5-fold [5.06 ± 0.85 (SE); n = 7] enrichment of 5′-nucleotidase and a 2.6-fold [2.60 ± 0.27; n = 9] enrichment of alkaline phosphodiesterase I activities in the membrane fraction compared with the starting homogenate.

EGF Binding to Bladder Membranes. The binding of 125I-EGF to bladder tumor membrane EGFr (Fig. 1) at 26°C was maximal after 15–30 min and remained stable for up to 180 min. All subsequent binding studies were carried out after 120 min incubation at 26°C.

Fig. 2A shows that the binding of 125I-EGF to a representative sample of bladder tumor membranes was competed for by unlabeled EGF in a dose-dependent manner. The 50% inhibitory concentration was 1.8 nm. No competition for binding sites was exhibited by α-melanocyte stimulating hormone, bombesin, adrenocorticotropic, or somatostatin. In a second experiment using a different sample of bladder tumor membranes the binding of 125I-EGF to EGFr sites was competed for by increasing concentrations of unlabeled TGF-β1, -3, and 5, and 5′-nucleotidase were both assayed according to the method of Aronson and Touster (18).
similar to that for unlabeled EGF (Fig. 2B).

When the data from Fig. 2A were transformed into a Scatchard plot (Fig. 3B), a single class of high affinity binding sites was detected. The dissociation constant (Kd) in this sample was 1.22 nM and the maximum number of binding sites (Bmax), 1040 fmol/mg protein, was calculated by linear regression analysis. This tumor had a high EGF receptor content but similar saturation kinetics and Scatchard analyses were obtained with other EGF positive tumors (Fig. 3D).

When the early time points of binding of 125I-EGF to bladder tumor membranes (Fig. 4) were plotted according to second order kinetics, a straight line was obtained (Fig. 4, inset) indicating the interaction between ligand and receptor to be of second order. The rate constant of association (k+1) was calculated from the equation

\[ t = \frac{2.303}{K_d(E_0 - R_0)} \log \frac{R_0}{E_0R_t} \]

where \( E_0 \) is the concentration of 125I-EGF, \( R_0 \) is the concentration of EGF receptor binding sites, \( E \) is the concentration of 125I-EGF at time \( t \), and \( R_t \) is the concentration of EGF at time \( t \). The value for the rate constant of association was 0.905 \times 10^9 M^{-1} min^{-1}.

The rate of dissociation of EGF from its receptor was assayed by incubating receptor-125I-EGF complexes with an excess of unlabeled EGF and then quantifying the receptor bound 125I-EGF at various times (Fig. 5). The half-life for dissociation of the receptor-EGF complex was 14 min. The points, when plotted according to first order kinetics (inset), fell on a straight line indicating a first order reaction. The rate constant of dissociation (k-1) was calculated from the equation

\[ k_{-1} = \frac{0.693}{t_{1/2}} \]

where \( t_{1/2} \) is the half-time for dissociation of 125I-EGF from the EGF receptor. The rate constant of dissociation was 0.0495 min^{-1}.

The measured rate constants can be substituted into the equation, \( K_d = k_{-1}/k_+ \) to give a value of 54.8 pM which is approximately 20-fold lower than the value of 1 nM calculated by equilibrium binding.

Quantification of EGF Binding to Bladder Tumors. The assayable EGF receptor content was determined in 24 muscle invasive (T2, T3, and T4) and 23 superficial (pTa and pT1) tumors and in 6 normal bladder samples using Scatchard analysis (Fig. 6). The Kd of the receptor ranged between 0.19 and 2.38 nM (mean, 0.94 nM; median, 0.79 nM) in the invasive and 0.2 and 1.45 nM (mean, 0.86 nM; median, 0.88 nM) in the superficial tumors. Owing to the numbers of samples assayed, it was decided to classify the tumors as invasive or superficial depending on the presence of muscle invasion. The results show the
highest receptor levels were in muscle invasive tumors. The concentration of receptor in muscle invasive tumors (99.9 ± 252.2 (SD) fmol/mg protein; median, 20.5) was greater than in tumors not invading muscle (12.4 ± 12.3 fmol/mg protein; median, 11.0) (P = 0.051, Mann-Whitney U test).

Moreover, when the 2 largest groups of superficial and invasive tumors were compared (15 pTa and 16 T3) it was found that the invasive tumors had greater levels of EGFr compared with the superficial tumors (P < 0.05, Mann-Whitney U test). Thus a subset of invasive tumors have a high EGFr level. If one looks at the data points in Fig. 6 it is clear that 2 of 23 superficial tumors versus 9 of 24 invasive tumors have levels of EGFr above 25 fmol/mg protein (the upper limit for normal bladder).

**Affinity Labeling of the Bladder Tumor Membrane EGFr.** Fig. 7 shows the results of cross-linking of 125I-EGF to a membrane preparation derived from a muscle invasive bladder tumor. Two high molecular weight proteins with molecular weights of approximately 150,000 and 115,000 are labeled. The labeling of the higher molecular weight species was slightly more intense and the receptor content was expressed as fmol specific binding/mg proteins.

**DISCUSSION**

The results presented in this study show that high affinity low capacity binding sites for EGF are present in membranes prepared from human bladder tumors. Physicochemically, the binding behavior was similar to that previously characterized in A-431 cells (21), placenta (22), and nonneuronal brain tumors (7), ovarian, cervical, and vulval tumors (13), and breast cancers (5, 6).

The EGFr receptor levels in all tumors were derived from Scatchard plots. In tumors classified as receptor negative, there was essentially no competition between increasing concentrations of unlabeled EGF and 125I-EGF, implying that the binding was of a nonspecific nature. In tumor samples with greater than mass to the high molecular weight affinity-labeled protein detected previously in cross-linking studies (Fig. 7), is observed. Addition of unlabeled EGF prior to phosphorylation (Fig. 8, Lane 1) results in markedly increased phosphorylation of this protein, suggesting that EGF is stimulating phosphorylation of the M, 160,000 species.

Addition of ethylene glycol bis(β-aminoethyl)ether)-N,N',N'-tetraacetic acid prior to phosphorylation (Fig. 8, Lanes 3 and 4) abolished labeling of the M, 160,000 species whether EGF was present or not. It was noteworthy that in these samples there was labeling at the origin of the gel suggesting that the phosphorylated EGFr and/or other phosphorylated products had possibly undergone a physicochemical reaction which prevented them from entering the gel during electrophoresis.

In the presence of calcium (Fig. 8, Lanes 5 and 6), two proteins, M, 160,000 and 125,000, were phosphorylated. Unlabeled EGF again stimulated phosphorylation, although this effect was confined to the M, 160,000 species. The total amount of phosphorylation was also greater when calcium was present.

Treatment of the gel with 1 m NaOH for 2 h at 55°C prior to autoradiography (Fig. 8, Lanes 7 and 8) shows the M, 160,000 phosphorylated protein to be relatively resistant to hydrolysis. Phosphotyrosine bands are resistant to alkali hydrolysis (20), implying that tyrosine residues are being phosphorylated.
in the presence of 15 \(^{\text{55P}}\) ATP. The ATP concentration used in that the molecular weight of the intact M, 174,000, EGFr could estimated substrate is of approximately the same size as the higher tyrosine kinase activity. There was a marked increase in the likely that this could explain the difference of approximately 160,000 and 125,000 were phosphorylated. Epidermal growth factor markedly enhanced phosphorylation of the M, 160,000 species but only marginally enhanced phosphophorlation of the M, 125,000 species. King and Gates (38) found that the mature EGFr in A431 cells was cleaved by a calcium-dependent enzyme (calcium activated neutral protease or CANP) to a lower molecular weight form of 150,000 which could still autophosphorylate; however, EGF-stimulated phosphophorylation of this form was only 10% of the autophosphorylation rate of the intact form of the receptor. Erneux et al. (39) have also showed that the calcium-dependent cleaved fragment of the receptor has a reduced autophosphorylation activity compared to the intact receptor. Gulick et al. (40) reported that the calcium-dependent enzyme, calpain, cleaved a fragment containing the 3 sites of autophosphorylation from the intact receptor. If a calpain-like enzyme is active in bladder tumor membranes, little or no EGFr stimulated phosphorylation of the M, 125,000 species would be expected due to loss of the major autophosphorylation sites but there are additional autophosphorylation sites on the receptor (41) which would explain the small degree of autophosphorylation of the M, 125,000 species.

There was considerable variation in the receptor content of the various tumors. It is unlikely that tumor heterogeneity is the explanation for our finding that muscle invasive tumors are more likely to be positive when studied by ligand binding compared with superficial tumors because two previous and independent studies have shown that muscle invasive tumors are more likely to be EGFr positive compared with superficial tumors when stained immunohistochemically (8, 11). Using the ligand binding assay, we also observed a similar result with invasive tumors having both a greater receptor content (\(P = 0.051\), Mann-Whitney \(U\) test) and a greater percentage of receptor positive tumors. When the two largest subgroups in the superficial and invasive tumors (pT1 and pT2) were compared, there was a significantly greater receptor content in the invasive tumors (\(P < 0.05\), Mann-Whitney \(U\) test). Two of the muscle invasive tumors had EGFr levels of 1040 and 791 fmol/mg protein, respectively, which were greatly in excess of the remaining tumors. While this would have raised the mean receptor content of the invasive tumors would not have affected the statistical significance of the difference between muscle invasive or superficial tumors because the Mann-Whitney test was used which is suitable for such nonparametric data and is based on the rank order of the result. To see whether the EGFr levels could be correlated with the EGFr gene copy number Berger et al. (11) performed Southern blot analysis on several of these bladder tumors. Interestingly, only 1 tumor, that containing 791 fmol/mg protein, showed gene amplification of the EGFr gene. Other transactivating factors may therefore be involved in regulating EGFr expression.

The presence of greater levels of EGFr receptor in invasive tumors of the bladder implies a possible role for the receptor in bladder tumor invasion. The survival rate of patients with muscle invasive bladder cancer is considerably less than those with superficial cancers (42, 43). Monitoring the long term survival of the patients in this study to see whether any correlation exists between the presence of EGFr receptor and time of survival will be very interesting and our present follow-up data3 suggests a relationship between the presence of the EGFr and

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3 Manuscript in preparation.
an adverse prognosis in both superficial and invasive tumors. Furthermore it will also be important to see if anti-EGFr antibodies or EGF linked drugs can be selectively targeted in vivo in bladder cancer patients.

This study has confirmed that receptors for EGF are present in human bladder cancer and that they are of high affinity and are functional in that they can be autophosphorylated in the presence of EGF. In addition a correlation was found between high tumor stage and high levels of receptor measured by ligand binding.

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