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# Binding Properties of Biotinylated Epidermal Growth Factor to Its Receptor on Cultured Cells and Tissue Sections

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A biotinylated derivative of murine epidermal growth factor (EGF) was prepared by covalent attachment of the terminal amino group of EGF to N-biotinyl-ε-aminocaproyl-N-hydroxysuccinimide. The stoichiometry of biotin incorporation was in the range of one biotin moiety per EGF molecule. The biotinylated EGF (biotinyl-ε-caproyl-EGF, BioEGF) binds to EGF receptors on intact Ehrlich ascites carcinoma (EAC) cells with an affinity similar to that of native EGF and displays the same mitogenic activity as EGF in a soft agar test system with normal rat kidney (NRK) cells. BioEGF was visualized on cultured cells and tissue sections of a head and neck tumour by commercial streptavidin/avidin detection systems. Cytochemical analyses of certain tumour forms can be easily performed using the BioEGF probe.

**Key words: EGF derivative, EGF receptor, cytochemical detection, clinical oncology, tumor marker**

Chemically modified growth factors or hormones have been used before to characterize receptor distribution in cell cultures [1] and tissue sections [2]. Characterization of the epidermal growth factor receptors (EGFR) with regard to their expression in different kinds of human tumours, such as breast cancer [3], bladder [4], cervix [5], gastric carcinomas [6], lung tumours [7], or head and neck carcinomas [8], is of increasing interest. It has been shown that in certain tumours prognosis is related to the EGFR status [3] and that growth of breast cancer cells is directly correlated with the number of EGFR sites [9]. Therefore, it is of biochemical and clinical interest to have a simple method for screening big groups of patients with regard to EGFR status. Additionally, a probe is needed for EGFR detection in small tumours or metastases where binding experiments with microsomal membranes [10] are not easily applicable.

Several attempts have been undertaken to modify the alpha amino group of the EGF molecule. The reactive $NH_2$ group was covalently linked to ferritin [11], peroxidase [12], fluorescein [13], and fluorescent lactalbumin [14]. There was also a report in

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which a biotinylated EGF was used to study receptor distribution in A-431 cells [15]. There are no reports indicating the usefulness of the probes for cytochemical detection of EGFR in vitro and in vivo.

In the present paper we describe the synthesis of a biotinyl-ε-caproyl EGF derivative. BioEGF binds like native EGF with high affinity to EGFR and has the same biological activity as native EGF. Initial cytochemical data show that this BioEGF may serve as a probe for detection of EGFR both in cells and in thin sections derived from tumour tissue.

## MATERIALS AND METHODS

### Materials

Murine EGF was isolated as described by Savage and Cohen [16] and has been characterized in binding studies before [9]. Streptavidin was a kind gift of Dr. Schroeder (Central Institut of Molecular Biology, Department of Chemistry). Radioiodination of EGF and streptavidin was performed by the chloramine-T method [17]. The specific activity of $^{125}$I-EGF or $^{125}$I-streptavidin was in the range of 8–10 or of 3–6 MBq per μg, respectively.

The reactive biotin derivative N-biotinyl-ε-aminocaproyl-N-hydroxysuccinimide (BioNHS) was synthesized according to Costello et al. [18], with an activation step of the carboxylic groups with isobutyl chloroformiate provided by Dr. Bauschke (Central Institute of Molecular Biology, Department of Chemistry). For the detection of BioEGF covalently coupled avidin-alkaline phosphatase (avidin-AP) from Sigma (St. Louis, MO) or the preformed complex streptavidin/biotinylated peroxidase (strep-POD) from Amersham (Buckinghamshire, UK) was used. The substrates diaminobenzidine (DAB) and o-phenylendiamine (OPD) were from Chemapol (Prague). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) were from Sigma.

### CELLS

Erlich ascites carcinoma (EAC) cells were collected from female mice 5–7 days after intraperitoneal transplantation of $2 \times 10^6$ tumour cells. A-431 cells and normal rat kidney (NRK) cells/clone 49F were continuously grown in Eagle's MEM, supplemented with 10% newborn or normal calf serum, respectively.

### Preparation of BioEGF

We first synthesized a biotinylated EGF using N-biotinyl-N-hydroxysuccinimide. This EGF-derivative, however, displayed a rather low intensity of staining in cytochemical experiments with A431 cells (not shown). In order to prevent a possible sterical hindrance of Bio-EGF-binding to its receptor in tissue sections, a biotin derivative containing a spacer arm (BioNHS) was introduced into the synthesis. In order to link EGF to BioNHS a technique described by Bonnard et al. [19] was applied, with several modifications as outlined below. EGF was dissolved to a final concentration of 0.10 mM in 50 mM phosphate buffered saline, pH 7.4 (PBS). BioNHS (22.8 mg per ml of dimethylformamide) was added at a 50-fold molar excess to EGF. For calculation of the yield of BioEGF in some experiments a tracer amount of $^{125}$I-EGF was added. The mixture was kept under stirring for 2 h at 23°C. The reaction was terminated by gel

filtration on Sephadex G-25 equilibrated with PBS. BioEGF eluted in the void volume. The BioEGF fractions were stored in aliquots at −20°C until use.

## Characterization of BioEGF

**Detection on nitrocellulose.** Detection of BioEGF on nitrocellulose was performed as follows: BioEGF or native EGF diluted in PBS at different concentrations was dotted on nitrocellulose. To reduce the nonspecific binding sites, strips were first incubated for 1 h at 23°C with 3% bovine serum albumin (BSA) in PBS, then washed five times for 5 min with a buffer consisting of 0.05% Tween 20 in PBS (detection with $^{125}$I-streptavidin or with the strep-POD complex) or with 0.1 M Tris-HCl buffer, pH 7.5, containing 2 mM $MgCl_2$, 100 mM NaCl, and 0.05% Triton X-100 ("washing buffer," detection with the avidin-AP complex). After the last washing, the nitrocellulose strips were incubated with different detection systems, i.e., $^{125}$I-streptavidin, strep-POD, or avidin-AP complex for 30 min at 23°C. The nitrocellulose was then washed and incubated five times for 5 min with either PBS containing 0.05% Tween 20 (in case of $^{125}$I-streptavidin or of strep-POD) or with 0.1 M Tris-HCl buffer, pH 9.5, containing 50 mM $MgCl_2$ and 100 mM NaCl ("substrate buffer," in case of avidin-AP). Finally, the nitrocellulose strips were processed for visualization by autoradiography or enzymatic staining as described [20,21].

**Evaluation of biotin incorporation.** In order to determine the relative amounts of free EGF, BioEGF was prepared as described above, adding $^{125}$I-EGF as a tracer. The radioactive BioEGF ($15–20 \times 10^3$ cpm) was mixed with a 25-fold molar excess of streptavidin. After a preincubation period of 30 min at 23°C, the resulting mixture was chromatographed on a Sephadex G-50 column, equilibrated with PBS. The column ($0.6 \times 40$ cm) was eluted at a flow rate of 10 ml/h. One milliliter fractions were collected and counted for radioactivity.

## Binding Experiments

The affinity of BioEGF for its receptor was evaluated by means of a competitive radioreceptor assay as described earlier [22]. In addition, BioEGF binding was followed using a receptor assay with A-431 cells. To this end cells were grown to confluence in 96-well plates. The cells were washed with 200 μl aliquots/well of PBS containing 1% BSA and then incubated with different BioEGF concentrations diluted in PBS/1% BSA in presence or absence of 1 μM native EGF for 60 min at 23°C. The incubation was terminated by aspirating the incubation medium from the cells. Then cells were quickly washed twice with 200 μl aliquots/well of cold PBS and incubated with 100 μl/well of a fixation medium consisting of 4% formaldehyde and 0.5% glutaraldehyde in PBS for 30 min at 4°C. In this way, 80% of bound $^{125}$I-EGF was covalently linked to A-431 cells (not shown). After fixation, cells were first washed with 200 μl aliquots/well of PBS and then with PBS/1% BSA. Then 100 μl/well of strep-POD complex diluted 1:500 in PBS/1% BSA were added. Cells were incubated for 30 min at 23°C and washed five times with 200 μl/well of PBS. Finally, 100 μl/well of 4 mM OPD substrate solution were added for 5 min. The reaction was terminated by addition of 30 μl/well of 1 M $H_2SO_4$ [23]. Absorption at 492 nm was measured and corrected by blank values of cells and reagents in absence of BioEGF. All values were estimated in triplicate. For comparison, binding of $^{125}$I-EGF to A-431 cells was measured under identical conditions except that cells were dissolved in 1 N NaOH and counted for radioactivity.

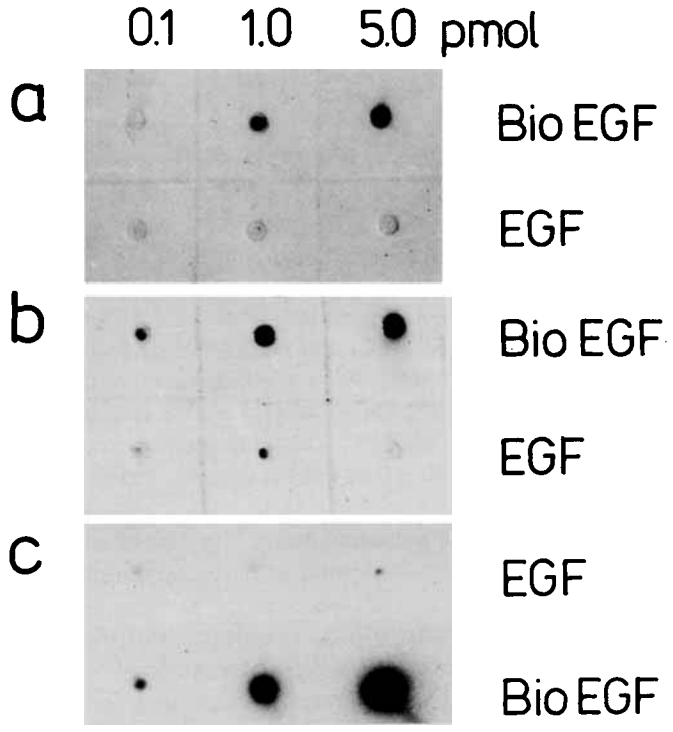


Fig. 1. Detection of BioEGF on nitrocellulose (**a**) enzymatic staining using the preformed strep-POD complex and DAB as the substrate. **b:** Enzymatic staining using the covalently linked avidin-AP complex and BCIP/NBT as substrates. **c:** Autoradiographic visualization showing the $^{125}$I-streptavidin binding. Time of exposure, 3 h. 0.1, 1.0, and 5.0 pmol of BioEGF were dotted onto nitrocellulose.

## Cytochemical Analysis

**Tissue sections.** Cytochemical analysis of tissue sections with BioEGF was performed essentially as described before [9]. Briefly, 5 μm sections were mounted onto glass slides and air-dried. The dried sections were washed twice with 1 ml aliquots of PBS/1% BSA and incubated in a total volume of 150 μl per section with 0.06–0.30 μg/ml of BioEGF dissolved in PBS/1% BSA for 2 h at 23°C. Control samples for nonspecific binding of BioEGF received an additional 60 μg/ml of native EGF. The sections were then washed twice with 1 ml aliquots of cold PBS and incubated with 1 ml of fixation medium. After fixation, the sections were washed twice with 1 ml aliquots of PBS and two times with 1 ml aliquots of "washing buffer." Then the sections were incubated with 150 μl per section of the "washing buffer" containing 1:1,000 diluted avidin-AP complex for 30 min at 23°C. Incubation was stopped by washing the sections again with 1 ml aliquots of this buffer, and then with 1 ml aliquots of the "substrate buffer." Finally, 150 μl substrate solution containing BCIP and NBT [21] were added to each section, and the reaction was stopped by addition of 0.1 M Tris/HCl buffer, pH 7.5.

**Cells.** A-431 cells were grown as monolayer on cover slides until confluence and processed as outlined above for tissue sections.

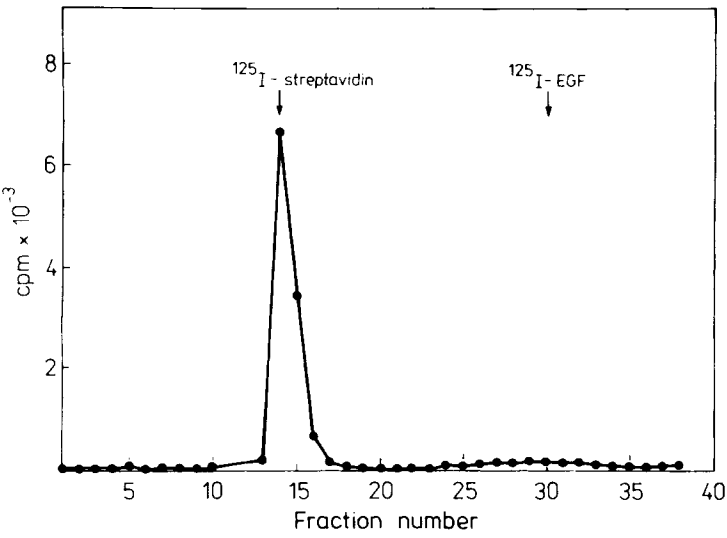


Fig. 2. Sephadex G-50 chromatography of the Bio$^{125}$I-EGF-streptavidin complex. The preformed Bio$^{125}$I-EGF/streptavidin-complex was chromatographed on Sephadex G-50 to remove noncomplexed ligands as described in Materials and Methods. 1 ml fractions were collected. The elution positions of uncomplexed $^{125}$I-streptavidin and of $^{125}$I-EGF are indicated.

## Biological Activity

The clonogenic activity of BioEGF was tested using NRK cells/clone 49F following the procedure described earlier [24].

## RESULTS

A biotin derivative of murine EGF was prepared using the reactive N-hydroxysuccinimidyl derivative of N-biotinyl-$\epsilon$-amino caproic acid, known to react with proteins at the $\alpha$- or $\epsilon$-$NH_2$ groups [19]. The BioEGF was detected with high specificity and sensivity by several streptavidin/avidin-based systems, as demonstrated in Figure 1. BioEGF dotted on nitrocellulose could be visualized by means of streptavidin/avidin-mediated enzymatic staining (Fig. 1a,b) or by $^{125}$I-streptavidin autoradiography (Fig. 1c). Best sensitivity was achieved with $^{125}$I-streptavidin detecting approximately 0.1 pmol BioEGF (Fig. 1c).

The stoichiometry of biotin incorporation into EGF molecules was evaluated by two indirect methods: First, the amount of BioEGF complexed by streptavidin was determined. To this end, a preformed complex composed of BioEGF prepared with tracer amounts of $^{125}$I-EGF (Bio$^{125}$I-EGF) and streptavidin was chromatographed on Sephadex G-50. The elution profile, shown in Figure 2, demonstrates the complete co-elution of Bio$^{125}$I-EGF with streptavidin, indicating a nearly 1:1 degree of substitution. Furthermore, the symmetrical shape of the streptavidin-Bio$^{125}$I-EGF peak excludes a remarkable dissociation of the preformed complex during the chromatographic separation (Fig. 2). Secondly, Bio$^{125}$I-EGF was passed over streptavidin covalently coupled to CNBr-activated Sepharose. Bio$^{125}$I-EGF was nearly quantitatively retarded, indicating also a complete substitution at the alpha $NH_2$ group of EGF (not shown). The

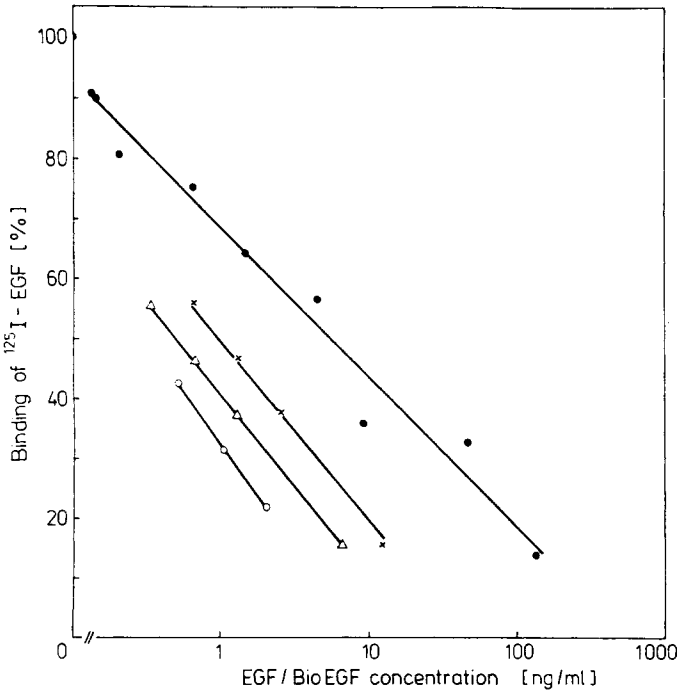


Fig. 3. Competitive radioreceptor assay. $^{125}$I-EGF was added to various amounts of unlabeled native (●) or biotinylated (△, ×, ○) EGF and incubated with EAC cells for 60 min at 23°C. Cells were centrifuged and cell-associated radioactivity was measured. Three independent preparations of BioEGF (○, △, ×) were compared.

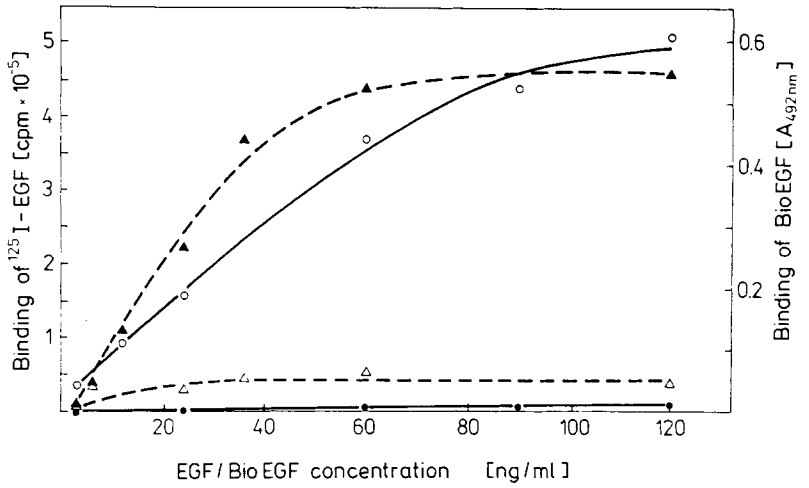


Fig. 4. BioEGF receptor assay. A-431 cells were incubated with various amounts of BioEGF in absence (▲) or in presence of 6 μg/ml of native EGF (△). After fixation, the covalently bound BioEGF was determined photometrically as described in "Materials and Methods. The strep-POD complex was used with OPD as the substrate. $^{125}$I-EGF binding in absence (○) or presence of 6 μg/ml (●) of native EGF was determined.

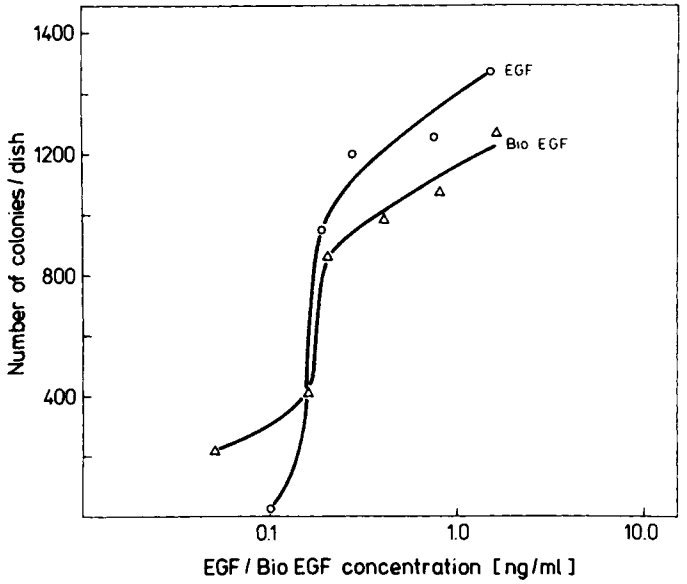


Fig. 5. BioEGF-dependent stimulation of growth of NRK cells in soft agar. $1 \times 10^4$ NRK-49F cells were seeded per 30 mm dish. After 8 days colonies greater than 50 $\mu$m were counted.

nonspecific binding of $^{125}$I-EGF to streptavidin-Sepharose was in the range of 10% of the total added radioactivity.

Next, the binding of BioEGF to the EGFR was investigated. BioEGF competed with $^{125}$I-EGF for binding to EGFR on intact EAC cells in a manner similar to that of native EGF (Fig. 3). The parallel slope of the binding curves indicates a similar binding affinity of BioEGF and EGF. The relative positions of the curves have to be interpreted with caution. They can be caused by erroneous calculation of BioEGF protein concentrations.

The specific BioEGF binding to A-431 cells was measured directly, by use of the strep-POD complex (Fig. 4). As shown, BioEGF binding became saturated at 60–80 ng/ml and was inhibited by EGF. For comparison, the saturation curve obtained with $^{125}$I-EGF is shown (Fig. 4).

In order to prove the mitogenic activity of BioEGF, a highly sensitive soft agar colony formation test with NRK cells was used. As shown in Figure 5, half-maximal stimulation of NRK cell colony formation was nearly identical for both BioEGF ($S_{50}$ = 0.18 ng/ml) and EGF ($S_{50}$ = 0.17 ng/ml).

For cytochemical demonstration of BioEGF binding sites, enzyme-linked detection systems employing strep-POD or avidin-AP were used. Figure 6a illustrates the detection of BioEGF bound to intact A-431 cells by means of the avidin-AP complex with BCIP/NBT as substrates. BioEGF staining was found over the cell surface with stronger intensity in the vicinity of neighbouring cells. In the appropriate control, containing an excess of unlabeled EGF, no staining was observed (Fig. 6b). Cryostat sections derived from a metastatic tissue of a head and neck epithelial carcinoma (Fig. 6d) were relatively strongly stained (Fig. 6c). Diffuse uniform staining was obtained on the tumor cells, whereas the infiltrating inflammatory cells were not stained.

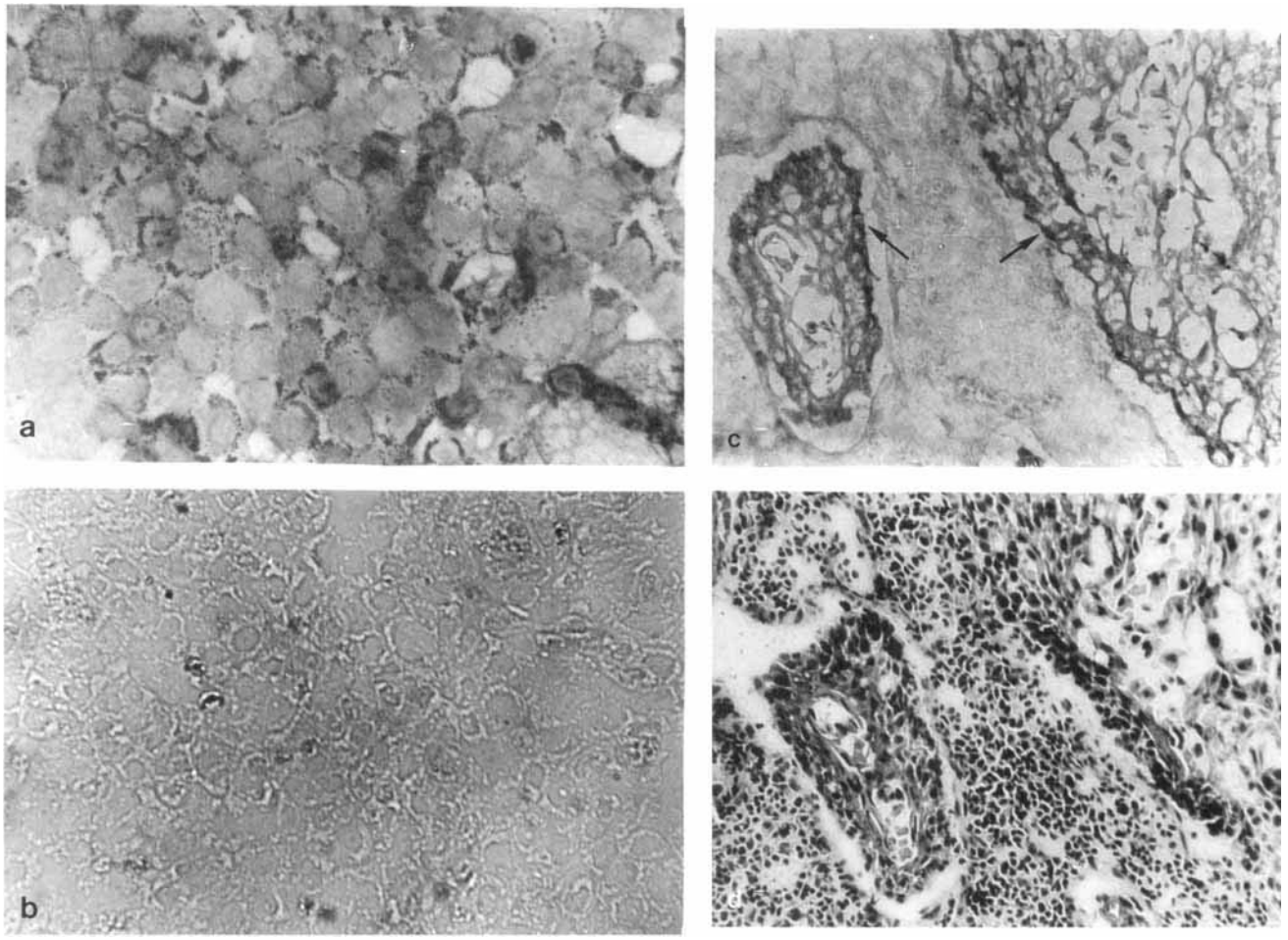


Fig. 6. Cytochemical demonstration of BioEGF binding. Cells grown on coverslides or tissue sections on glass slides were incubated with BioEGF. After washing and fixation covalently bound BioEGF was visualized by means of the avidin-AP detection system with BCIP/NBT as substrates. **a:** A-431 cells. **b:** Control for nonspecific BioEGF binding to A-431 cells obtained in presence of an excess native EGF. **c:** 5 $\mu$m sections obtained from a metastasis of a head and neck epithelial carcinoma. The arrows indicate tumour cell clusters. **d:** Consecutive section from the same tumour sample after hematoxylin-eosin staining. a–d, $\times 100$.

## DISCUSSION

In this report we describe a novel synthesis of a biotinylated EGF using a reactive biotin derivative with ϵ-aminocaproic acid as a spacer arm. The synthesis resulted in a biologically fully active BioEGF in high yield. The BioEGF binds with the same affinity as EGF to EGFR and stimulates NRK cells to grow in soft agar at concentrations nearly identical with those found with EGF. The reactive biotin derivative N-biotinyl-ϵ-aminocaproic acid we used ensured a high affinity of streptavidin binding to EGF [19]. This was necessary in order to use the probe for detection of EGFR in A-431 cells and in thin sections of tumour tissue. The staining pattern was similar to that observed with a monoclonal antibody directed to the binding domain of the EGF receptor (R. Perez et al., unpublished results). However, nonspecific background staining occasionally occurring in immunocytochemical studies is avoided when BioEGF is used for receptor labeling. So far, our histochemical data prove that BioEGF detects EGFR in tissue sections of a receptor-rich tumour. Now it is possible to perform a more detailed study to evaluate the spectrum of tumours accessible to an analysis with BioEGF. In this respect, BioEGF could be a valuable probe for characterization of very small tumours or metastases which cannot be analyzed by the conventional biochemical ligand binding assay [10]. BioEGF binds with same affinity as native EGF to its receptor and both stimulate DNA synthesis at $10^{-10}$ M. Therefore, the probe should be useful in studying EGFR interactions and the ligand-receptor fate. Labeling of EGF by cross-linking the molecule to either ferritin or peroxidase [11,12] or by converting EGF into a fluorescent derivative [13,14] resulted in EGF probes of decreased biological activity. Moreover, the yield was much lower than with BioEGF. Better results were obtained by Guigni et al. [15] using N-biotinyl-N-hydroxysuccinimide for the synthesis of biotinylated EGF. The authors mentioned that this probe was biologically active; however, data documenting its use in cytochemistry were not given. Our attempts to use it in cytochemistry were not successful (E. Spitzer, unpublished results). In the light of the potential clinical importance of the EGFR status as a prognostic marker [3], more clinical data on EGFR expression in malignant tumours of different origin are desirable. BioEGF presented in this report can be useful in clinical cytochemistry for evaluating the EGFR status in series of patients.

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