A CYTOTOXIC EPIDERMAL GROWTH FACTOR CROSS-LINKED TO DIPHTHERIA TOXIN A-FRAGMENT

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1. Introduction

In order to better understand the molecular and genetic mechanisms of cellular responses to bioactive polypeptides, such as peptide hormones and growth factors, through interaction with specific receptors [1–3] we have been undertaking an approach using the strategy of mutagenesis [4,5]. We have devised a new selection procedure to isolate insulin receptor-deficient mutants by using a cytotoxic insulin cross-linked to diphtheria toxin A-fragment and proposed a possible mechanism of cell killing [6,7]. Recently, this technique has enabled us to isolate several variants from mouse Swiss/3T3 fibroblasts which have lost insulin binding ability and insulin responsiveness ([8], submitted).

Here, we have applied the crosslinking method to a potent mitogen, epidermal growth factor (EGF, M_r 6045) [2]. Fragment A of diphtheria toxin (DTa; M_r 21 150) [9] was coupled via a disulfide bond to a cystamine-derivatized EGF. The crosslinked molecules were purified by Biogel P-10 column chromatography. The purified EGF–DTa conjugate was shown to inhibit the binding of ^{125}I-labeled EGF to cell surface receptors. It revealed strong cytotoxicity to human epidermoid carcinoma cells, A431, possessing a high number of EGF receptors while it was ineffective to mouse NR-6 cells which are EGF receptor-deficient variants from Swiss/3T3 fibroblasts. These data strongly suggest that the EGF–DTa conjugate delivers catalytically active toxin A-fragment into A431 cells through EGF receptor-mediated endocytosis and inactives elongation factor 2 by ADP-ribosylation.

2. Materials and methods

Diphtheria toxin A-fragment was prepared as in [6]. Epidermal growth factor was purified from mouse submaxillary glands by the method in [10]. EGF–DTa conjugation was done as in [6] with slight modification. Briefly, EGF (0.8 mg) was dissolved in 1 ml 50 mM acetic acid and dialyzed against 2 changes of distilled water containing 1 μg phenylmethyl sulfonyl fluoride (PMSF)/ml, 500 ml each. Cystamine dihydrochloride (225 mg) was added, dissolved and the pH was adjusted to 4.7 with 1 N HCl. 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide–HCl (EDAC, 1.53 mg) was added and the pH maintained at 4.7 for 10 min with gentle stirring. The reaction mixture was then dialyzed against 2 changes of 20 mM *N*-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES) (pH 6.5), 500 ml each, to remove unreacted cystamine dihydrochloride and EDAC. Diphtheria toxin A-fragment (1 mg) was reduced by incubating with 1 ml of 0.1 M dithiothreitol (DTT) at 4°C for 4 h and then dialyzed against 1.5 l 20 mM TES (pH 6.5). About 0.6 ml reduced fragment A was added to 1 ml cystamine–EGF derivative and the pH was adjusted to 7.4. The mixture was dialyzed against 1.5 l of 20 mM TES (pH 7.4) containing 1 μg/ml PMSF for 18 h at 4°C to allow formation of spontaneous disulfide bonds.

2.1. *Purification of EGF–DTa conjugate*

The conjugate (0.8 ml) was applied onto a column (1.4 × 28 cm) of Biogel P-10 pre-equilibrated with 0.15 M NaCl in 50 mM HCl. The materials were eluted first with the same buffer, secondly with 20 mM TES (pH 7.4) and finally with 4 M urea in 20 mM TES (pH 7.4). Fractions of 1.4 ml were collected.

2.2. *Inhibition of ^{125}I–EGF binding*

Cell cultures were maintained in Dulbecco's modified Eagle (DME)-medium supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) under 5% CO_2–95% air and humidified atmosphere at 37°C. Confluent cell cultures in 60 mm diam. plastic petri dishes were placed on ice and washed twice with ice-cold EBSS buffer, 2 ml each, as in [5]. The washed cells were then covered with 1 ml EBSS buffer containing the desired amounts of EGF–DTa conjugate and incubated for 10 min at 23°C. Then, 10 μl ^{125}I-labeled EGF (150 μCi/μg, 10 μCi/ml, Collab. Res.) were added at 1.1×10^{-10} M final conc. After incubation for an additional 60 min at 23°C, the plates were washed 3 times with ice-cold EBSS buffer, 3 ml each. The remaining radioactivity was counted as in [5]. Human epidermoid carcinoma cell line A431 [11] was obtained from Dr S. Cohen and mouse cell line NR-6 [12] was kindly provided to us by Dr H. Herschman.

2.3. *Killing of cells*

This was done by adding EGF–DTa conjugate (0.002 A_{280} units) into logarithmically growing cells in 35 mm diam. dishes. The change of morphology was monitored under a Nikon inverted phase-contrast microscope and photographed according to the manual.

3. Results and discussion

Highly pure DTa retains its ability to inactivate elongation factor 2 by ADP-ribosylation [9]. Each of such DTa fragments contains an SH-group which can be reacted with cystamine-derivatized EGF to form a disulfide bond at pH 7.4 (see section 2). When the reaction product was analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS) (fig.1A) two major bands, I and II, appeared in addition to a strong band corresponding to unreacted DTa and a faint, broad band corresponding to EGF-cystamine derivatives. Incubation of the reaction mixture with 2-mercaptoethanol eliminated bands I and II, revealing only the two bands co-migrating with DTa and EGF-cystamine derivatives (fig.1B). The broad appearance of the latter band is presumably due to heterogeneity of cystamine-treated EGF. This is expected since EGF contains several carboxyl groups [2] which could have reacted with

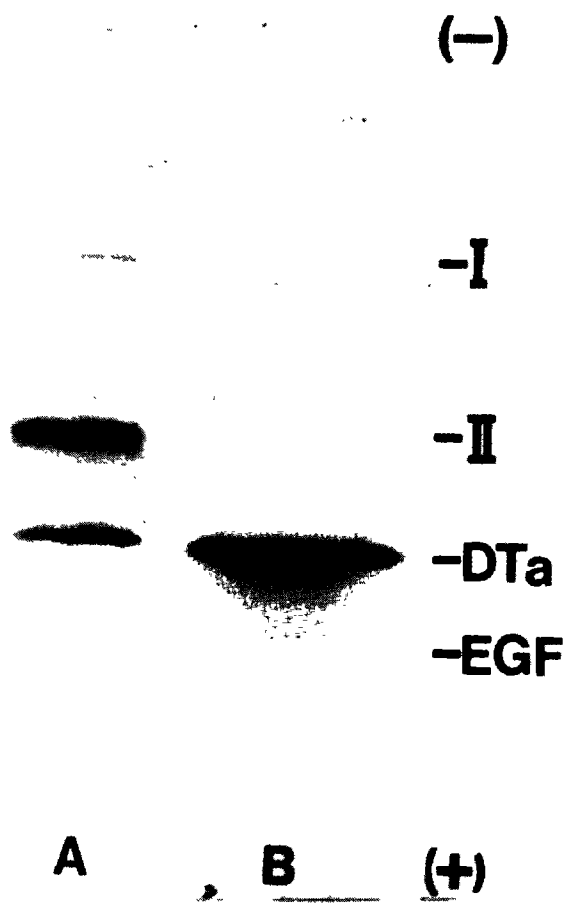


Fig.1. SDS–polyacrylamide (10%) gel electrophoresis of conjugation products, showing two components of crosslinked molecules. Before (A) and after (B) treating with 2 mercaptoethanol to cleave disulfide bonds. DTa and EGF indicate the migration positions of these markers in a separate channel. The electrophoresis conditions were 100 V, 18 mA/slab gel for 135 min by the method in [15].

cystamine. These data clearly indicate the presence of disulfide linkage in the molecules of band I and band II.

The reaction mixture was then loaded onto a column of Biogel P-10 and the materials eluted successively with 3 different elution buffers as is illustrated in fig.2. Each eluate was dialyzed to remove salts and analyzed by a SDS–polyacrylamide gel electrophoresis under the same conditions as fig.1. Peak 1 appeared at the position of 1.8 column vol. at which EGF is supposed to be eluted as observed by others under identical conditions [10]. Analysis of peak 1 material on the polyacrylamide gel in fact revealed a broad band corresponding to EGF-cystamine derivatives. Peak 2 contained a single component with mobil-

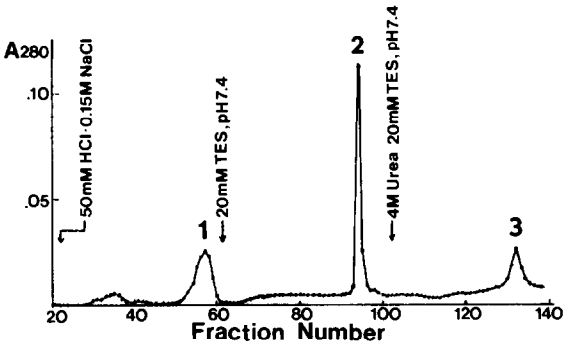


Fig.2. Purification of conjugation products by Biogel P-10 column chromatography.

ity identical to that of band II. After reduction of this peak 2 material, two bands were detected which correspond to EGF-cystamine derivatives and DTa. Peak 3 appeared to be a mixture of band I and band II materials. From these data we conclude that peak 2 material is a conjugate of cystamine-derivatized EGF and DTa, linked via disulfide bond and free from EGF or its cystamine-derivatives. According to their migration positions, it is probable that band I is either a DTa dimer or $(DTa)_2$–EGF and that band II is a 1:1 conjugate of DTa and EGF.

The purified EGF–DTa conjugate, peak 2 material, was tested for its ability to compete with ^{125}I-labeled intact EGF for binding to cell surface receptors. Inhibition of binding is clearly seen in fig.3, demonstrating that the EGF moiety of the conjugate recognizes specific EGF receptors expressed on the surface of human and mouse cells. About 0.0003 A_{280} units of conjugate was needed to obtain 50% inhibition on mouse 3T3 cells under the binding conditions used. About 7-times more conjugate was required to gain the same degree of inhibition on human A431 cells. This is consistent with the observations that A431 cells express an extraordinarily high number of EGF receptors [11,13].

Human cells are generally more susceptible to intact diphtheria toxin (DT) than are mouse cells and can be killed since DT or DTa, which has been uptaken into cells, shuts off protein synthesis in a highly specific and efficient manner [9]. DTa fragments are not cytotoxic to cells because they are incapable of entering cells due to a lack of cell surface binding sites [9]. Our preparation of pure DTa did not contain any trace of intact DT [6] and did not interfere with the growth of A431 cells. Human A431 cells, however, showed apparent morphology changes when they were cultivated in the presence of pure EGF–DTa conjugate (fig.4). Cell fragmentation occurred within 24 h and the destruction continued with further incubation. Within 4–6 days all of the cells had died. We have tested another cell line, NR-6, which is of mouse origin and is deficient in EGF receptors [12]. When NR-6 cells were treated in the same manner as A431 cells they grew normally to confluency. Thus, cell killing is related to the presence or absence of specific EGF receptors.

We have described the synthesis of a cytotoxic EGF crosslinked to the catalytic fragment of diphtheria toxin and demonstrated that its cytotoxicity is dependent upon specific EGF receptors. These data

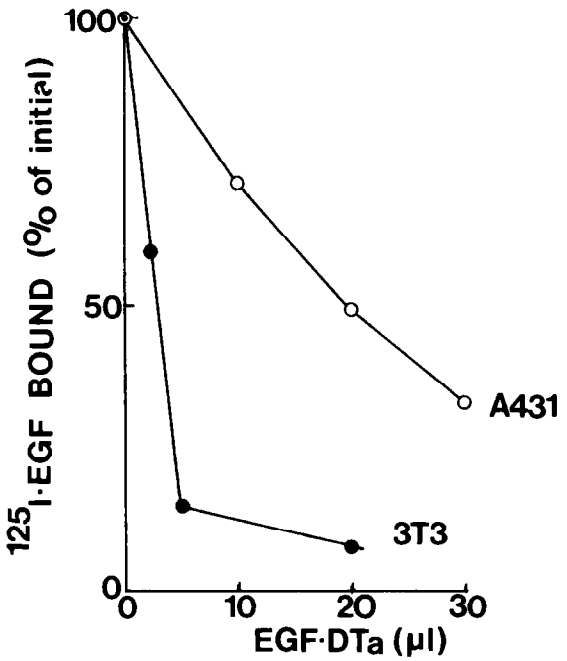
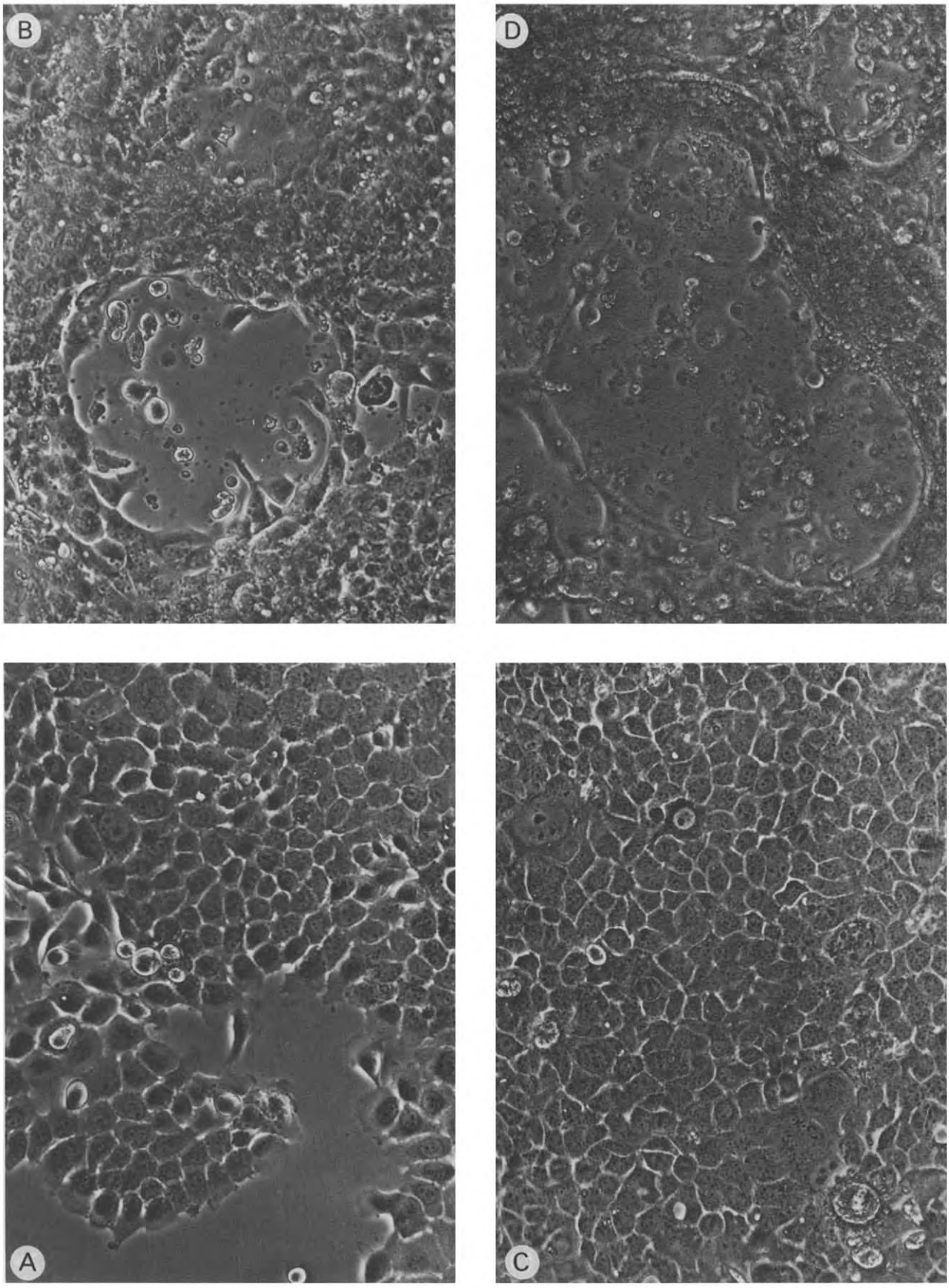


Fig.3. Inhibition of ^{125}I-EGF binding to human A431 and mouse 3T3 cells by the EGF–DTa conjugate. Confluent monolayer cultures were treated with the indicated volume of the conjugate solution (0.112 A_{280} units/ml) for 10 min at 23°C and then incubated with ^{125}I–EGF for an additional 60 min. The cells were extensively washed and the remaining radioactivity counted.

Fig.4. Killing of human epidermoid carcinoma cells, A431 line, by the EGF–DTa conjugate. Subconfluent A431 cells were treated with 0.001 A_{280} units/ml conjugate for 24 h (B) and for 48 h (D). Drastic cell destruction can be seen compared to the control cultures, (A) for 24 h and (C) for 48 h.


B
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provide additional evidence to our proposal [6,8] that a bioactive polypeptide with a relatively small molecular weight can be crosslinked to DTa without loss of receptor recognizing activity and serve as a delivery vehicle of the toxic fragment A into viable cells. In this context, it should be noted that specifically bound EGF is internalized by A431 cells and processed through the lysosomal pathway [13,14]. The EGF–DTa conjugate is being used as a selection agent for mutants which are defective in EGF–receptor functions in order to provide them for further study of the genetic basis of EGF–receptor interactions [5].

Acknowledgements

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