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EPIDERMAL GROWTH FACTOR RECEPTORS IN THE HUMAN GLIOBLASTOMA CELL LINE SF268 DIFFER FROM THOSE IN EPIDERMOID CARCINOMA CELL LINE A431

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SUMMARY: Two established human tumor cell lines, epidermoid carcinoma line A431 and glioblastoma line SF268, were studied to compare the interaction of each with epidermal growth factor (EGF). SF268 cells bound [^{125}I] EGF with 35-40fold higher affinity than did the A431 cells. The EGF binding sites of both lines were photoaffinity labeled using 2,4-NAPS-[^{125}I] EGF, a photoreactive derivative of EGF. Extracts of photolysed cells analyzed by SDS-PAGE showed a difference between the two cell lines in the high molecular weight component corresponding to the EGF receptor. EGF in a dose range from 0.3-200 nM had no effect on thymidine incorporation by SF268 cells, whereas thymidine incorporation by A431 cells was markedly inhibited by EGF.

Many normal cell lines and cell lines derived from malignant tumors contain receptors for epidermal growth factor (EGF) (1,2). The high level of expression of EGF receptor in the human epidermoid carcinoma cell line A431 (3-5) has allowed cloning and sequence analysis of the EGF receptor mRNA (6). Although there is little question that the EGF receptor mediates the mitogenic stimulus of EGF, the mechanism of transduction is not apparent from the molecular structure obtained (6). In A431 cells, EGF can have either positive or negative effects on proliferation, depending on the dose of EGF and the subclone of A431 used (7,8). In addition, the EGF receptor is a target for modulation by factors that regulate cell growth, including polypeptide growth factors and tumor-producing agents (9). Other cell lines with high levels of EGF receptors but with different functional properties, either more tightly

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Abbreviations: cpm, counts per minute; ED_{50}, effective mean dose; EGF, epidermal growth factor; HPLC, high pressure liquid chromatography; [^{125}I], iodine-125; MEM, mimimum essential medium; 2,4-NAPS-Cl, 2-nitro, 4-azidophenylsulfenyl chloride; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel elecrophoresis; SEM, standard error of the mean; TCA, trichloroacetic acid; UV, ultraviolet.

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coupled to mitogenesis or uncoupled from mitogenesis, might serve as useful models for understanding transduction in the EGF receptor. Radioiodinated EGF has served as a useful probe of receptor structure through the study of binding alone (10), or by covalent attachment using chemical crosslinking or photoaffinity labeling (11,12).

We have evaluated a series of human glioblastoma cell lines to determine the presence or absence of EGF receptors and their response to various growth factors in comparison to A431 cells (unpublished data). One human glioblastoma cell line, SF268, had high levels of EGF binding and appeared to have a higher affinity toward EGF than did A431. Using radioiodinated EGF and a novel photoreactive derivative of EGF, we further characterized the nature of differences between the EGF binding sites in A431 and SF268 cells. Our findings are summarized in this report.

MATERIALS AND METHODS

Cell Culture. A431 cells were obtained from the Cell Culture Facility, University of California, San Francisco. The SF268 cell line (13) originated from a patient with a glioblastoma multiforme. Cells from passages 32-36 were maintained in T75 culture flasks (Falcon, Becton-Dickinson, Oxnard, California) or on 24 well or 6 well plates (Costar, Cambridge, Massachusetts) at 37° C in an 8% CO_2 humidified atmosphere using modified Eagle's medium with Earle's balanced salt solution, 20% fetal calf serum (Hyclone Sterile Systems, Logan, Utah), and 50 µg/ml gentamycin (Schering, Kenilworth, New Jersey).

Iodination of EGF. EGF was isolated from mouse submaxillary glands in the manner described by Cohen and Savage (14). The isolated material was more than 95% pure by amino acid analysis and reverse phase HPLC. EGF (7 µg) was iodinated with 1 mCi [^{125}I] NaI (New England Nuclear, Boston, Massachusetts) by the Chloramine-T method (15). Iodinated EGF ([^{125}I] EGF) was separated from free ^{125}I by gel filtration on a 0.6 x 20 cm column of Sephadex LH-20 (Pharmacia, Priscataway, New Jersey) equilibrated with 1.0 M pyridine, 0.5 M acetic acid (pH 5.5). Fractions containing [^{125}I] EGF were stored at -20° C after addition of an equal volume of glycerol.

Binding Experiments. Cells grown to confluence in 24 well plates were washed once with cold PBS and 1 ml of binding buffer (PBS, pH 7.4; 10 mg/l $CaCl_2$; 10 mg/l $MgSO_4$; 0.1% bovine serum albumin) was added. EGF (0-1 µM) was added followed by 250,000 cpm [^{125}I] EGF. Each dose of EGF was assayed in quadruplicate. After 2 hr of incubation at 4° C, the buffer was removed and the cultures were washed once with cold PBS. Cells were then lysed by incubation in 0.5 M NaOH at 37° C for 60 min; the lysate was transferred into glass tubes for gamma counting. Three wells chosen randomly from each experimental plate were spared for protein determination by the Lowry method (16).

Preparation of 2,4-NAPS-[^{125}I] EGF. EGF was iodinated as just described, except that the initial separation was performed in 90% acetic acid. Under dim room light, 2 mg of methionine and 2 mg of 2,4-NAPS-Cl (17) were added to the fraction containing the most [^{125}I] EGF. After 2 hr incubation at 23° C in the dark, 2,4-NAPS-[^{125}I] EGF was separated from reagent by gel filtration at room temperature in the dark on a 0.6 x 20 cm column of Sephadex LH-20 equilibrated with 5% acetic acid, 10% ethanol, and was used immediately for photoaffinity labeling.

Photoaffinity Labeling. Cells were grown to confluence in 35-mm 6 well culture plates (Costar). Binding experiments using 2,4-NAPS-[^{125}I] EGF as radioligand were performed in the dark as just described; unlabeled EGF was added to alternate wells. After 2 hr at 4° C, the incubation medium was aspirated and one plate of each cell line was left in the dark; another was exposed to UV light (Blak-Ray UV lamp, model B-100 A, Ultraviolet Products, San Gabriel, California) twice for 12 min at a distance of 10 cm from the lamp. Cell layers were extracted with 1.0 ml of 0.0625 M Tris-HCl (pH 6.8) containing 2% SDS, 8 M urea, and 0.001% bromphenol blue. The extracts were heated at 95° C for 10 min and stored at 4° C until used for further analysis. Unirradiated cells were washed and extracted in the dark, but thereafter were treated in the same manner as the photolysed cells.

SDS-PAGE. The buffers described by Laemmli (18) were used. Molecular weight standards (Biorad, Richmond, California) or cell extracts (20 µl) were applied to duplicate lanes of a 2-mm slab of 4.5% acrylamide (stacking gel) and 8% acrylamide (stacking gel). A current of 20 mA was applied. The gels were fixed, stained with Coomassie Blue R250, and dried. Gels were exposed to XAR-5 film (Eastman Kodak, Rochester, New York) for 1 week at -70° C with a Lightning Plus intensifying screen (DuPont, Wilmington, Delaware).

HPLC-Gel Filtration. Extracts (0.5 ml) from photoaffinity labeling experiments were applied to a TSK 4000 GSW 0.75 x 30-cm column equilibrated with 0.1% SDS, 50 mM $NaPO_4$ (pH 7.4); 0.5-ml fractions were collected at a flow rate of 0.5 ml/min and then counted for 1 min on a gamma counter.

DNA Synthesis. A431 and SF268 cells were plated at an initial density of 10^4 cells per 10-mm well. While still subconfluent, their medium was changed to serum-free MEM for 24 hr. The medium was changed again and fresh serum-free medium was added. At the same time, EGF was added in final concentrations ranging from 0.3-200 nM. After 20 hr, tritiated thymidine (10^6 cpm per well; New England Nuclear; 49 Ci/mmol) was added. After 2 hr, the medium was aspirated and the cells were washed twice with 500 µl of cold PBS per well. The cell layer was then treated with cold TCA, washed once more with PBS, and then incubated in 250 µl of 0.2 M NaOH for 60 min at 37° C. The lysate was transferred into liquid scintillation vials. Lysate from a second wash of individual wells with 100 µl of 0.5 M HCl was also added. After the addition of scintillation fluid (Hydrofluor; National Diagnostics, Sommerville, New Jersey) and equilibration for 30 min, the samples were counted on a liquid scintillation counter. DNA synthesis is assumed to be proportional to the radioactivity incorporated into this TCA insoluble fraction.

RESULTS AND DISCUSSION

Binding of [^{125}I] EGF to A431 and SF268 cells in confluent cultures showed that both cell lines have high levels of EGF binding. The amount of EGF binding to SF268 per culture well was about 35% of that binding to A431; when normalized with respect to total protein, EGF binding to SF268 cultures was approximately 80% of that binding to A431 cultures. However, the affinity of EGF binding was 38fold higher in SF268 cells than in A431 cells (Fig. 1). In three experiments, the ED_{50} for binding of [^{125}I] EGF to SF268 was 0.45 nM (0.3-0.85 nM); in A431 cells it was 17 nM (12.2-21 nM).

Photoaffinity labeling revealed marked differences between A431 and SF268 cells (Fig. 2). Four distinct bands were labeled in the A431 cells; the most

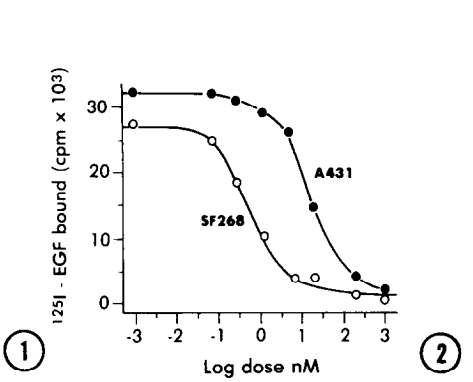

FIG. 1. [^{125}I] EGF binding to A431 cells (-●-) and SF268 cells (-o-) in culture. Confluent 10-mm cultures were incubated with 250,000 cpm [^{125}I] EGF and increasing doses of cold ligand for 2 hr at 4° C. Each point represents the mean of triplicate or quadruplicate cultures. Results are normalized with respect to protein content.

FIG. 2. SDS-PAGE analysis of photoaffinity labeled A431 extracts (1-8) and SF268 extracts (13-20). Dark controls (1-4, 13-16); photolysed (5-8, 17-20); no cold EGF (1, 2, 5, 6, 13, 14, 17, 18); 1 µg EGF (3, 4, 7, 8, 15, 16, 19, 20). [^{125}I] NAPS-EGF irradiated in the presence of A431 cells (9).

prominent band corresponded to a molecular weight of approximately 170,000. In the SF268 cells, labeling was observed in a broad region corresponding to a molecular weight range of 150,000 to 250,000. No labeled bands were seen in either cell line when high affinity binding was blocked by coincubation with unlabeled EGF (Fig. 2). Analysis of the cell lysates on a TSK 4000 gel filtration column in a HPLC system yielded similar results. In the elution profiles, the radioactivity associated with the most prominent peak obtained from A431 cells eluted more sharply and 3 fractions later than did the high molecular weight activity of SF268 (Fig. 3).

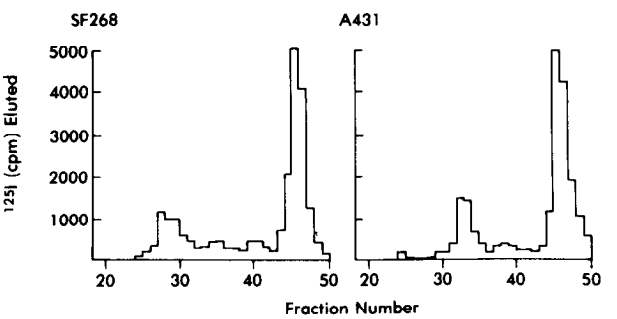

FIG. 3. HPLC-gel filtration analysis of photoaffinity labeled A431 and SF268 extracts. No radioactivity was detected in the unirradiated controls before fraction 44.

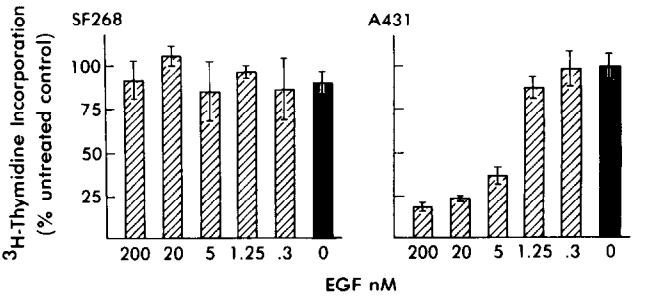

FIG. 4. DNA synthesis in cultures of A431 and SF268 after 20 hr of incubation with decreasing doses of EGF in serum free conditions. The mean (± SEM) incorporation of ^{3}H-thymidine in quadruplicate cultures during a 2-hr incubation with the nucleotide is expressed as a percentage of the value obtained for untreated control cultures.

The two cells lines also differed in their biological response to EGF. DNA synthesis in A431 cells was inhibited by higher concentrations of EGF. The addition of EGF over the same dose range (0.3-200 nM) had no effect on DNA synthesis by SF268 cells under identical experimental conditions (Fig. 4).

Our results thus show differences between these two human tumor cell lines with respect to EGF binding affinity, EGF receptor molecular weight and heterogeneity, and biological response to EGF. These differences could mirror normal physiological differences in the tissues in which the tumors developed (19). Alternatively, they may represent pathological differences related to malignant transformation. Although the structural basis of the differences is not known, it is clear that the EGF receptor from SF268 offers an attractive candidate for further comparative studies with A431 and placental EGF receptors. Insight into both the mechanism of signal transduction of the EGF receptor and the contribution of the receptor to malignant transformation might be obtained from an understanding of the structural differences between the homogeneous, biologically coupled, lower affinity, lower molecular weight receptors from A431 and the heterogeneous, uncoupled, higher affinity, higher molecular weight receptors from SF268.

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REFERENCES

1. Carpenter, G., and Cohen, S. (1979) Ann Rev Biochem 48, 193-216.
2. Gospodarowicz, D., Moran, J.S. (1976) Ann Rev Biochem 45, 531-558.
3. Fabricant, R.N., DeLarco, J.E., Todaro, G.J. (1977) Proc Natl Acad Sci (USA) 74, 565-569.
4. Haigler, H., Ash, J.F., Singer, S.J., Cohen, S. (1978) Proc Natl Acad Sci (USA) 75, 3317-3321.
5. Gill, G.N., Lazar, C.S. (1981) Nature 293, 305-307.
6. Ullrich, A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A., Tam, A.W., Lee, J., Yarden, Y., Libermann, T.A., Schlessinger, J., Downward, J., Mayes, E.L.V., Whittle, N., Waterfield, M.D., Seeburg, P.H. (1984) Nature 309, 418-425.
7. Buss, J.E., Kudlow, J.E., Lazar, C.S., Gill, G. (1982) Proc Natl Acad Sci (USA) 79, 2574-2578.
8. Kawamoto, T., Mendelsohn, J., Le, A., Sato, G.H., Lazar, C.S., Gill, G. (1984) J Biol Chem 259, 7761-7766.
9. Davis, R., Like, B., Massague, J. (1985) J Cell Biochem 27, 23-30.
10. Carpenter, G., Cohen, S. (1976) J Cell Biol 71, 159-171.
11. Das, M., Miyakawa, T., Fox, C.F., Pruss, R.M., Aharonov, A., Hershmann, H. (1977) Proc Natl Acad Sci (USA) 74, 2790-2794.
12. Sahyon, N., Hock, R.A., Hollenberg, M.D. (1978) Proc Natl Acad Sci (USA) 75, 1675-1679.
13. Rosenblum, M., Gerosa, M.A., Wilson, C.B., Barger, G.R., Pertuiset, B.R., De Tribolet, N., Dougherty, D.V. (1983) J Neurosurg 58, 170-176.
14. Cohen, S., Savage, C.R., Jr. (1975) Methods in Enzymology 37, 424-430.
15. Hunter, W.M., Greenwood, P.C. (1962) Nature 194, 195-196.
16. Lowry, O.H., Rosebrough, N.J., Farr, A.L. (1951) J Biol Chem 193, 265-275.
17. Muramoto, K., Ramachandran, J. (1980) Biochemistry 19, 3280-3286.
18. Laemmli, U.K. (1970) Nature 227, 680-685.
19. Westermark, B., Magnusson, A., Heldin, C-H. (1982) J Neurosci Res 8, 491-507.