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CHARACTERIZATION OF EPIDERMAL GROWTH FACTOR RECEPTOR IN A RAT THYROID CELL LINE, FRTL-5

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A rat thyroid cell line FRTL-5 showed no significant binding of epidermal growth factor(EGF) either in the presence or absence of thyrotropin(TSH). Conditioned medium from FRTL-5 did not inhibit the specific binding of [^{125}I]EGF to HeLa cell which has EGF receptors (EGF-R). Two bands of 170KD and 80KD obtained by immunoprecipitation with solubilized FRTL-5 and anti-EGF-R monoclonal antibody indicated autophosphorylation activity. The activity was enhanced by several thyroid cell growth factors including TSH, dibutyryl-cAMP(db-cAMP), insulin like growth factor I(IGF-I), Interleukin 1β(IL-1β) and phorbol ester. EGF alone enhanced the autophosphorylation activity. These data indicate that FRTL-5 possesses unique EGF-R with low affinity to EGF and that the tyrosine kinase of the receptor is activated during cell proliferation.

Epidermal growth factor (EGF) is a growth factor of the thyroid cell and has an inverse relationship between binding affinity to the receptor and EGF-induced cell proliferation(1). FRTL-5 is a rat thyroid cell line, which maintains several thyroid functions(2). Recent data indicate that the cell does not show any significant binding to EGF nor does EGF facilitate growth of the cell line(3). In this study, we analyzed EGF-R of FRTL-5 and found several findings: 1)no significant binding to EGF was observed, 2)detection of proper EGF-R is possible by immunoprecipitation with anti-EGF-R monoclonal antibody, 3)several thyroid growth factors (TSH, db-cAMP, IGF-I, IL-1β, TPA and EGF) modulate the autophosphorylation activity of EGF-R.

MATERIALS AND METHODS

1. Cells

FRTL-5 cells(ATCC No. CRL8305) were cultured in Ham's F-12 medium supplemented with 10 ng/ml somatostatin, 10 μg/ml insulin, 5 μg/ml transferrin, 10 ng/ml glycyl-histidyl-L-lysine, 10 nM hydrocortisone, 0.3 mU/ml bovine TSH and 5% calf

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serum (6H-5CS)(2). HeLa(ATCC No. CCL2), 3Y1 (Fisher rat fibroblast) and A431 cells(human epidermoid cell) were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum(DME-10F).

2. Reagents

EGF and IGF-I were purchased from Toyobo C.(Tokyo, Japan). TGFα, transferrin, bTSH, db-cAMP and 12-O-tetradecanoyl-phorbol-13-acetate(TPA) were purchased from Sigma(St. Louis, MO). IL-1β was provided by Genzyme Corp.(Boston, MA). A monoclonal antibody against human EGF-R which cross-reacts specifically with the EGF receptor of mouse, rat, guinea pig, rabbit and monkey was purchased from Transformation Research Inc.(Framingham, MA). The antibody shows four bands in an immunoblotting assay using human A431 cell.

3. EGF binding study and analysis of EGF-like substance from FRTL-5

EGF binding study was performed as described previously(4). HeLa cells were cultured at 4 x 10^5 cells per dish and EGF binding studies were performed as described above. Four day-cultured conditioned medium from FRTL-5, TGFα and EGF were previously incubated for 60 min or added simultaneously with [^{125}I]EGF to this system, and final cell bound radioactivities were compared.

4. Immunoprecipitation

FRTL-5, 3Y1 and A431 cells(5 x 10^6) were metabolically labeled with 0.1 mCi/ml of L-[^{35}S] methionine(Amersham, UK) for 20 hr. They were then rinsed twice with PBS and solubilized with HNTG buffer(20 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1mM EGTA, 10 μg/ml aprotinin(Sigma)(5). The solutions were incubated for 30 min at 4 ℃ with anti EGF-R monoclonal antibody conjugated with protein G-Sepharose (Pharmacia Fine Chemical, Uppsala, Sweden). Unbound materials were washed out four times with HNTG. The bound protein was subjected to 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by autoradiography.

5. Autophosphorylation activity of EGF-R of FRTL-5

Autophosphorylation studies of EGF-R were performed by the method of Kris(5). Briefly, FRTL-5(6 x 10^6) were cultured in 6H-5CS for 48 hr. After rinsing once with PBS, cells were cultured in Ham's F-12 containing 1%BSA and 10ng/ml transferrin for 48 hr. Subsequently, the cells were cultured with various thyroid cell growth factors including bTSH(0.3, 10 mU/ml), db-cAMP(0.1, 1mM), IGF-I(1, 10 ng/ml), IL-1β(1, 10 U/ml), TPA(100 ng/ml), EGF(0.1, 1, 10 nM) for 4 hr. Then the cells were collected and solubilized in HNTG buffer. EGF-R were collected as described above, and incubated with adenosine 5'-(γ-^{32}P) triphosphate(Amersham, UK) at 4 ℃ for 10 min. They were then subjected to SDS-PAGE and exposed to an X-ray film. After development, the densities of the bands were measured with a Laser Densitometer(Pharmacia LKB, Sweden).

RESULTS AND DISCUSSION

1. Binding of EGF to FRTL-5

Rat fibroblast cell lines, 3Y1 and FRTL-5 are both derived from the Fisher rat. As shown in Fig.1, [^{125}I]EGF showed specific binding to 3Y1, but did not bind to FRTL-5. Specific binding of EGF to thyroid cells has been reported to increase in the presence of TSH(6). But the addition of 0.3 mU/ml bTSH could not induce any specific binding of EGF to FRTL-5. Three possible mechanisms could explain these phenomena: (A)absence of EGF receptor of FRTL-5 cells, (B)existence of EGF receptor with a mutated form of EGF binding site, (C)occupied EGF receptor with EGF-like substances secreted from FRTL-5 cells.

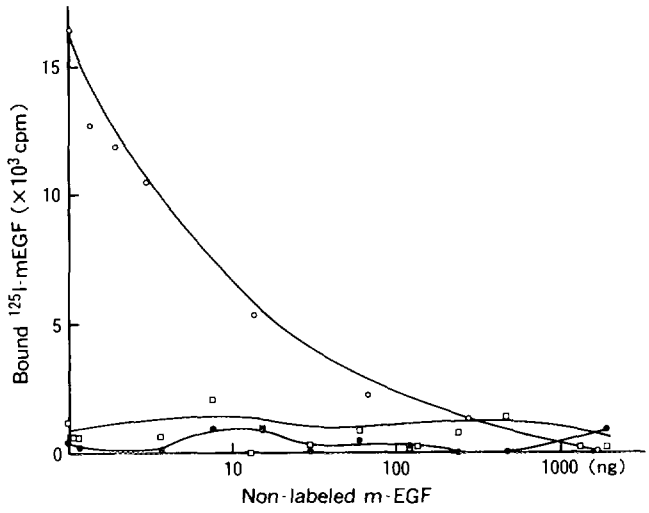

Figure 1.
Binding study of [^{125}I]EGF to FRTL-5.
FRTL-5 cells(●) or rat fibroblast cell line, 3Y1(○), derived from Fisher rat, were cultured without 0.3 mU/ml bTSH for 48 hr, and then incubated with [^{125}I]-labeled and non-labeled EGF. Addition of TSH(□) did not induce significant EGF-binding in FRTL-5.

2. Immunoprecipitation using a specific monoclonal antibody against EGF-R

Several bands of molecular size 56K - 170K(FRTL-5), with two additional bands of 100K and 97K(3Y1), several from 80K to 170K(A431, positive control) were seen(Fig. 2). The highest molecular weight EGF-R which immunoprecipitated from both FRTL-5 and 3Y1 was 170k, which is in agreement with the generally recognized molecular weight of EGF-R(7). Moreover, Northern blotting analysis using pE7(a cDNA probe for intracellular domain of v-erbB which has high homology to EGF-R gene(8)) also supported the existence of EGF-R mRNA with the predicted size in FRTL-5(data not shown).

3. No secretion of EGF-like substance from FRTL-5

As shown in Table 1, specific [^{125}I]EGF binding to HeLa cells was effectively displaced with non-labeled EGF and TGFα with a common binding site to EGF-R(9). In the case of FRTL-5-conditioned medium, however, no significant decrease of bound radioactivity was observed. Even when the conditioned medium was previously incubated with the cells, [^{125}I]EGF binding to HeLa was not inhibited. An immunoprecipitation study with mouse monoclonal antibody against EGF-R, however, showed the existence of the receptor in FRTL-5 with the expected molecular weight. This rules out a possible autocrine mechanism by an EGF-like substance, and suggests an aberrant binding site for EGF.

4. Autophosphorylation activity of EGF-R in FRTL-5

After determining the presence of EGF-R, it was necessary to determine whether or not the biological activity of EGF-R is preserved. We examined whether or

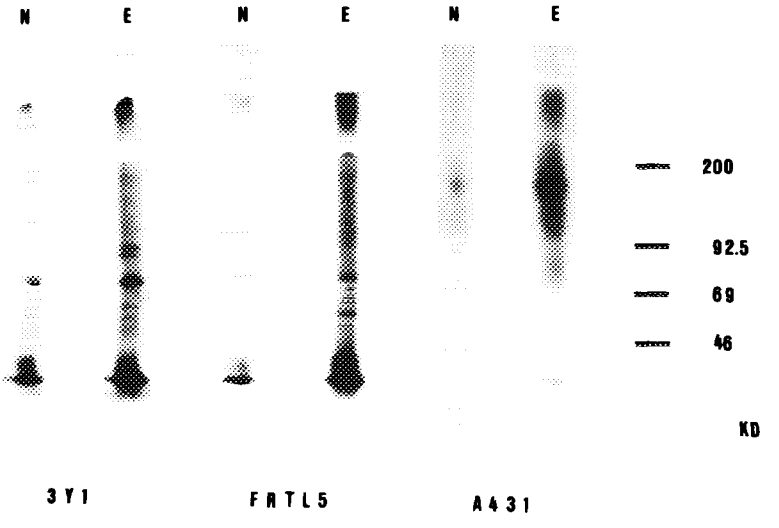

Figure 2.
Immunoprecipitation of EGF-R.
FRTL-5, 3Y1 and A431 cells were cultured in the presence of L-[^{35}S] methionine for 20 hr. Then the solubilized cell membranes were incubated with normal mouse serum(N) or anti-EGF-R monoclonal antibody(E). The immune complex were collected with protein G-Sepharose and applied to SDS-PAGE. The gel was exposed to a X-ray film for 2 days. Bars show the positions of radioactive molecular markers.

not the immunoprecipitated bands from FRTL-5 have tyrosine kinase activity. In the absence of any growth factors except transferrin, 2 weak bands of 170k and 80k were observed(Fig. 3A). Furthermore, the addition of growth factors such as TSH, db-cAMP, IGF-I, IL-1β and TPA to FRTL-5 cells enhanced the phosphorylation of both bands to a similar extent (Fig. 3B). To our surprise, EGF also induced the enhancement of autophosphorylation of EGF-R. This data indicated that the EGF receptor of FRTL-5 has tyrosine kinase activity and preserves autophosphorylation activity. TSH is the most potent growth factor for FRTL-5. Its binding to thyroid cells activates the adenylate cyclase, and controls the production of c-AMP and regulates thyroid cell functions and proliferation(10). In the absence of TSH, the immunoprecipitated EGF-R showed low autophosphorylation activity; but the addition of TSH enhanced tyrosine kinase activity and increased the receptor autophosphorylation by 2.6-4.4 times. The phosphorylated bands were 170K and 80K, matching well with the molecular weight of EGF-R.

Insulin and IGF-I also induce thyroid cell proliferation(11). The site of action of IGF-I is thought to be different from the site of TSH, because synergism of TSH and IGF-I is reported during [^{3}H] thymidine incorporation into FRTL-5(12). When FRTL-5 was cultured with 0.3 mU/ml bTSH and 1 ng/ml IGF-I, the

Table 1
Effects of EGF, TGFα and FRTL-5-conditioned medium on the binding of radioactive EGF to HeLa cells

EGF(ng)	TGFα(ng)	FRTL-5-medium(μl)	Bound [^{125}I]EGF(cpm)
(a) Simultaneous Incubation with:			
0	0	0	18487.0
50	0	0	1598.0
0	50	0	8202.8
0	0	400	18293.7
0	0	700	20286.2
(b) Pre-incubation with:			
50	0	0	2652.0
0	0	440	16006.5
0	0	770	17684.5

Four-day cultured conditioned medium from FRTL-5 was added to HeLa cells previously or simultaneously with [^{125}I]EGF. As positive controls, non-labeled EGF and TGFα were incubated.

autophosphorylation of EGF-R was 1.4 times the effect of 0.3 mU/ml bTSH alone(data not shown). TPA has similar properties as EGF in augmenting thyroid cell growth and differentiation, inhibiting iodine uptake and decreasing the intracellular accumulation of c-AMP(13). When TPA(100 ng/ml) was added to FRTL-5, the autophosphorylation of EGF-R was 3.8 times higher than in the absence of any growth factors. This also suggests the close relationship between PI turnover, C-kinase action and thyroid cell growth. Interleukin 1β is another growth factor of the thyroid cell which also promotes synergistic DNA synthesis with IGF-I(14), although IL-1β alone has only slight stimulatory effects on thyroid cell proliferation. The addition of IL-1β alone to FRTL-5 showed enhancement of the autophosphorylation activity of EGF-R. Thus, the level of autophosphorylation activity of EGF-R was not equal to the cell proliferation,

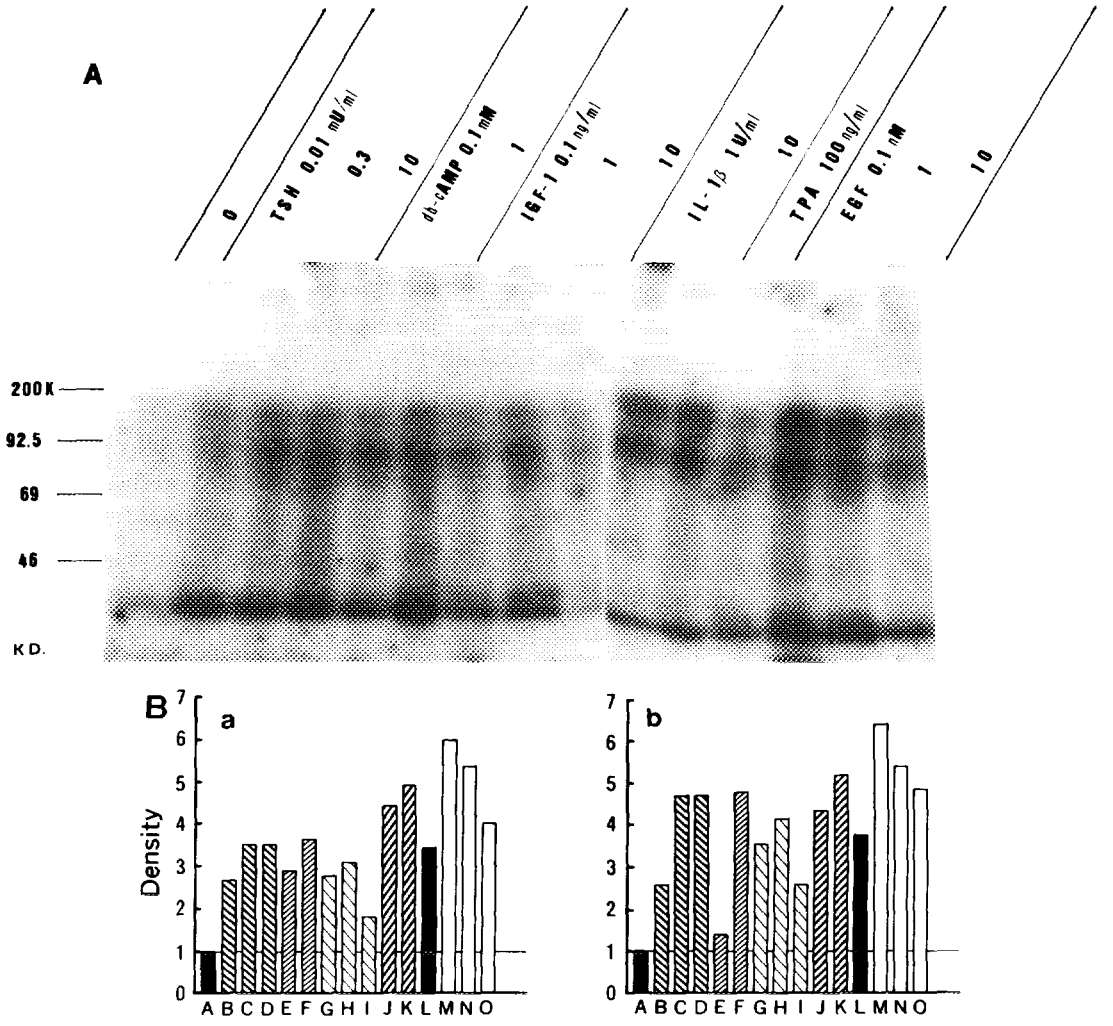

Figure 3.
Autophosphorylation of EGF-R in FRTL-5 cell.
(A)Autoradiography.
FRTL-5 cells were cultured with various substances for 24 hr. Bars show the position of molecular markers. See details in Materials and Methods.
(B)Analysis of phosphorylated bands by a laser densitometer.
X-ray film was applied to a laser densitometer, and the densities of each band (170 KD : a, 80 KD : b) were measured. For comparison of the autophosphorylation activities of EGF-R, the density of the band in the basal condition(A) was temporally designated as 1.0. The order of samples is same as in Figure 3A: 0.01, 0.3, 10 mU/ml TSH(B-D), 0.1 and 1 mM db-cAMP(E,F), 0.1, 1, 10 ng/ml IGF-I(G-I), 1 and 10 U/ml IL-1β(J,K), 100 ng/ml TPA(L), 0.1, 1 and 10 nM EGF(M-O).

although all thyroid growth factors studied enhanced the autophosphorylation activity. It suggests that the autophosphorylation of EGF-R is closely related to the process of the thyroid cell growth, but that phosphorylated EGF-R alone

may not be sufficient for cell proliferation and another signal transduction pathway may act with the enhancement of EGF-R autophosphorylation activity. Similarly, Schreiber et al.(15) reported that a cyanogen bromide cleaved analogue failed to induce DNA synthesis, but phosphorylation of endogenous membrane proteins mediated by the EGF specific, cyclic-nucleotide independent protein kinase activity was enhanced. Uncharacteristically, our results indicate that the addition of EGF also enhanced the tyrosine kinase activity of the receptor. The binding assay system we have used is sensitive enough to detect specific binding to the level of 10^{-6}M. Our results indicate the existence of an EGF-R with an aberrant binding site and low affinity.

There have been several recent reports concerning mutated EGF-R; mutation of ATP binding site of EGF-R(16), K-ras transformed cell line with an improper insertion of EGF-R into the cell membrane(17) and variants of 3T3 without EGF-binding nor mitogenic response to EGF(18). The last mutant might have aberrant EGF-R similar to FRTL-5. It is not clear how the process of acquisition of thyroid specific differentiation functions during the establishment of FRTL-5 occurs, but the lack of normal EGF-R must allow FRTL-5 to maintain this process. Complex events proceed between EGF binding and thyroid cell growth as described above. Molecular analysis of EGF-R of FRTL-5 will evaluate the possible mutation of EGF binding site of EGF-R. If it is not case, DNA-mediated gene transfer experiment of authentic EGF-R gene to FRTL-5 will answer the question whether non-mitogenic effect of EGF and aberrant binding of EGF to FRTL-5 are due to aberrant EGF-R-linked machinery or aberrant post-transcriptional modification of EGF-R. The actual process from the binding of EGF to the receptor to the final cell growth event is still unclear, but the unique characteristics of EGF receptor of FRTL-5 with tyrosine kinase activity modulated by thyroid cell growth factors may provide a new tool to further study cell growth facilitation by EGF.

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