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Elevated Expression of Epidermal Growth Factor Receptors Mediated by Retinoic Acid in Human Lung Cancer Cells

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ABSTRACT

In human lung cancer cell line, H460, an accumulation of epidermal growth factor receptor (EGF-R) was observed following treatment with 1 μM of retinoic acid. An increase in ^{125}I-labeled EGF binding capacity was detected after 48 h incubation with retinoic acid. Increased autophosphorylation of EGF-R after 48 h treatment of retinoic acid treatment correlates with enhancement of EGF binding capapcity on H460 cell surface. When treated cells were plated in retinoic acid-free medium after 72 h treatment with retinoic acid, EGF-R expression level remained intact. This phenomenon represents a novel EGF-R synthesis on cell surface , induced by retinoic acid.

Keywords: human lung cancer cells, epidermal growth factor receptor, retinoic acid

Introduction

Human epidermal growth factor receptor (EGF-R) is expressed in various carcinoma cell lines and tumor tissues (Carpenter and Cohen, 1990). Human tumor progression is associated with the expression of EGF-R via gene amplification or transcriptional expression level escalation (Liberman *et al.*, 1984; King *et al.*, 1985; Yamamoto *et al.*, 1986). Regulation of EGF-R expression is reportedly regulated by various agents, e.g. phorbol-myristate acetate (Jetten *et al.*, 1981; Saito *et al.*, 1982). Retinoic acid has been reported associated with the induction of the EGF-R in normal fetal rat lung cells by elevating its mRNA expression (Thompson *et al.*, 1989). Retinoic acid is known to be a significant effector in normal and malignant cells growth modulation (Jiang *et al.*, 1990). The increasing EGF binding capacity in several cell types after retinoic acid treatment has also been found (Yung *et al.*, 1989). We report here that retinoic acid treatment results in posttranscriptional elevation in EGF-R expression. The resulting EGF-R enhancement increases tumorigenicity phenotype of human lung cancer cells.

Methods and Materials

Cell cultures

The cell line used, H460, is a clonal derivative of the human non-small cell lung cancer cell line. The cell line was acquired from Dr. A. Gazdar of the Southwestern Medical Center, Dallas, TX. The cells were maintained in RPMI-1640 supplemented with 5% fetal calf serum and were free of mycoplasma contamination. In treated H460 cells, 1 μM of retinoic acid (Sigma, St. Louis, MO) was added in cultures and incubated at 37 ℃ in humidified 5% CO_2.

Immune complex kinase assay for expressed EGF-R

Kinase assays were performed as previously described (Maxwell *et al.*, 1989). Cells were lysed and Dounce-homogenized in RIPA lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM ethylenediamine-tetraacetic acid (EDTA), 1% aprotinin, 5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, and 20 mM sodium phosphate, pH 7.0). Five hundred micrograms of clarified cell lysates were incubated for 1 h with 5 μl of a monoclonal antibody against EGF-R extracellular domain, R_1, (Amersham, Arlington Heights, IL). Immune complexes were harvested by addition of *Staphylococcus aureus* (Cowan strain) (Calbiochem, La Jolla, CA) for 30 min. A buffer containing 10 μl [γ^{32}P] adenosine-5'-triphosphate (ATP) , 6 mM manganese chloride , 20mM sodium *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonate (HEPES) (pH 7.0), and 10 μM sodium orthovanadate was added. The reaction was terminated with RIPA buffer and the mixture washed with RIPA buffer three times. Phosphorylated proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 7.5% resolving gels). The gels were washed in 1 N NaOH at 80℃ for 1 hand

dried. The dried gels were exposed to Kodak X-Omat film for 1 h before development.

^{125}I-labeled EGF binding assay

Receptor grade EGF (Collaborative Research, Boston, MA) was labeled with carrier-free Na^{125}I (ICN, Irvine, CA.) using chloramine-T method (Carpenter and Cohen, 1976). Retinoic acid-treated H460 cells were washed with phosphate-buffered saline (PBS) that contains 0.2% bovine serum albumin (BSA) and replaced with serum-free medium for an additional 3 h. Various concentrations of ^{125}I-labeled EGF were added to each well. After a 2 h incubation at 4℃, the cells were washed three times in ice-cold PBS, and the bound radioactivity was determined after the cells were lysed in a 50 mM NaOH and 10% SDS mixture. The nonspecific binding was determined in assays containing a 100-fold molar excess of native EGF. Quantitation of binding sites per cell, and the dissociation constant, K_d, was determined by Scatchard analysis (Scatchard, 1949). The A431 human epidermoid cancer cell line was used as control.

Biosynthetic labeling in intact cells

Immunoprecipitation was performed as previously described (Steck *et al.*, 1986; Honegger *et al.*, 1990). In brief, cells were cultured in 1 μM retinoic acid supplemented medium for the specified time before labeling. The growth medium was removed, and cells were incubated for 1 h in methionine-free RPMI-1640 containing 10% dialyzed fetal calf serum and 50 μCi ^{35}S-methionine (1,100 Ci/mmole; ICN, Costa Mesa, CA). After labeling, cells were washed with PBS and extracted with a mixture of 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM ethyleneglycol-*bis*-(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton-X, 4 μg/ml PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 100 mM NaCl, 10 mM sodium pyrophosphate and 30 mM p-nitrophenyl phosphate. Monoclonal antibody R_1 was added to individual cell lysate with equal radioactivity counts, followed by 1.5 h incubation. Fifty microliters of *Staphyloccous aureus* was added for 30 min at ice cold temperature. After washing, pellets were extracted in SDS-PAGE sample buffer, heated to 100℃ for 3 min, and applied to vertical slab gels. For fluorography, gels were treated with Enlighting (New England Nuclear-Dupont) before drying. Dried gels were exposed to Kodak X-Omat film at -70 ℃ before development.

Tumorigenicity in nude mice

Viable H460 cells were treated with 1 μM retinoic acid for 72 h. The cells were then trypsinized and washed with PBS. The cells (2×10^5) were mixed in Hanks Balanced Salt Solution and inoculated subcutaneously in five 6-week old female *nu/nu* mice. The cross-sectional diameters of the resulting tumors were measured externally every five days for 35 days. Tumor volume was calculated according to the published procedure (Mukhopadhyay, *et al.*, 1991).

Result

^{125}I-labeled EGF binding assays

The effect of retinoic acid on the affinity of EGF binding capacity was determined by increasing labeled EGF in retinoic acid-treated and -untreated H460 cells Human epidermoid cells were used as control. The dose-titration binding analysis of H460 cells showed that EGF binding capacity increases after retinoic acid treatment (Fig. 1b). The EGF binding capacity increase was detected within 24 h after treatment. The numbers of binding sites were shown increased by more than 40-fold after 48 h treatment and dropped off after further treatment (Fig. 1a, Table 1). Nearly 25% of cell surface EGF binding sites that appeared after 48 h were lost after further 24 h treatment There exists only one type of EGF-R in H460 cells and was not altered during retinoic acid treatment.

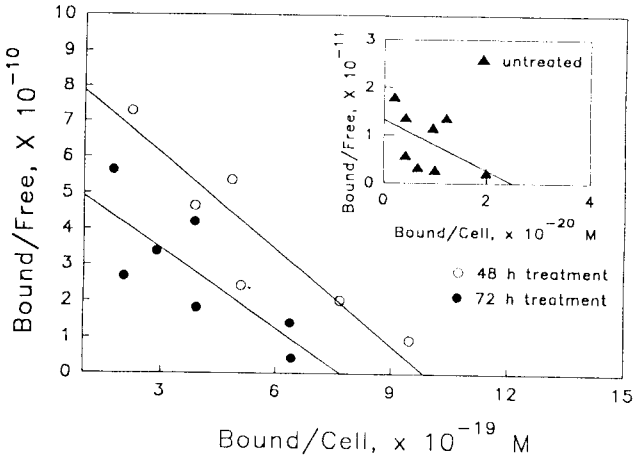
Table 1 . Analysis ^{125}I-labeled EGF binding properties of EGF-R expressed in retinoic acid-treated H460 cell at different time points.

Type	High Affinity		Low affinity	
	K_d (nM)	N ($\times 10^5$)	K_d (nM)	N ($\times 10^5$)
A431	0.6	11	8	30
H460	—	—	0.8	0.14
H460 (48h)	—	—	1.09	5.4
H460 (72h)	—	—	1.13	4.2

Note. ^{125}I-labeled EGF binding was assessed over a range of concentrations from 0-50 ng/ml of EGF in triplicate wells as specified in Materials and Methods. Specificity of binding was determined in parallel competition experiments using a 100-fold excess of unlabeled EGF. The dissociation constant, K_d, and the numbers of EGF binding sites per cell, N, were derived from Scatchard analysis (Scatchard, 1949). Human epidermoid cells A431 was used as positive control.

EGF-R autophosphorylation assay

The degree of phosphorylation of EGF-R was determined by immunoprecipitation with extracellular domain specific antibody, R_1, followed by incubation with [γ^{32}P]ATP. The level of EGF-R autophosphorylation increases after retinoic acid treatment. The activity reached maximum after 48 h treatment (Fig. 2, lane 3) and declined thereafter (Fig. 2, lane 4). That corresponds with increases of EGF binding capacities following treatment,rather than enhanced phosphorylation rates of



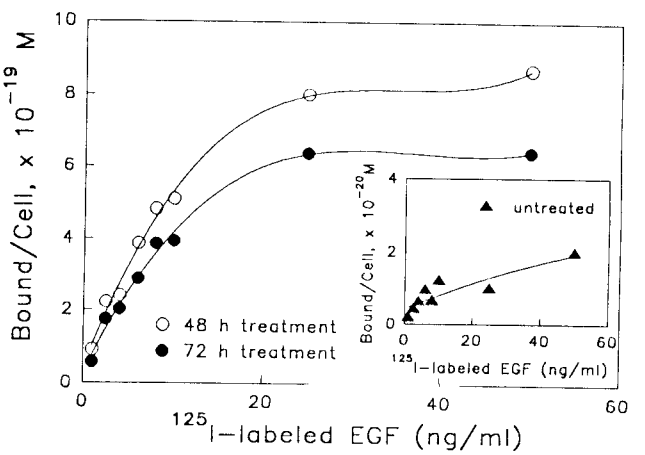


Fig.1: ^{125}I-labeled EGF binding anaylsis. ^{125}I-labeled EGF (0-50 ng/ml) in PBS was added to 5 × 10^5 of retinoic acid-treated or -untreated cells in 60 mm petri dishes. Binding capacity was determined after incubation at 4 °C for 2 h. Non-specific binding was determined after addition of a 100-fold excess of unlabeled EGF and was always less than 3% of the total binding. Scatchard analysis was used to determine binding sites and dissociation constants, K_d (a). The binding capacity with increasing ^{125}I-labeled EGF concentration was indicated in the bottom panel (b). The binding of ^{125}I-labeled EGF to A431 cells as controlled set was performed at the same time that exhibited two types of binding sites. Cells treated for 0, 48 and 72 h are represented by (▲), (○) and (●,inset), respectively.

EGF-R. Cell line A431 was used as control (Fig. 2, lane m).

Time course expression of EGF-R via metabolic labeling in intact cells

Fig. 2: Immune complex kinase assay for EGF-R Cell lysate in RIPA buffer was incubated in EGF-R ligand binding specific antibody R_1. Immune complexes were harvested by addition of *Staphylococcus aureus* and incubated with [γ^{32}P]ATP. Phosphorylated proteins were separated by SDS-PAGE gel (7.5%). The gels were washed in 1 N NaOH at 80°C and dried before exposure. In the parallel control set, A431 cells were incubated with R_1 antibody (lane m).

To evaluate the formation of EGF-R in *in vivo* system, the cells were labeled metabolically with ^{35}S-methionine. After solubilization, the cell lysates were immunoprecipitated with EGF-R extracellular domain specific antibody, R_1, and equal radioactive of cell lysate was analyzed with SDS-PAGE gels. The results indicated that EGF-R formation escalated with increasing time, and, after apexed at 48 h of retinoic acid treatment (Fig. 4, lane 5), declined thereafter. The level of EGF-R expression remained unchanged in drug-free medium for another 24 h after 72 h treatment (Fig. 3, lane 7).

Tumorigenicity in *nu/nu* mice

Cells pretreated with retinoic acid for 72 h were inoculated subcutaneously in *nu/nu* mice and tumor formation was observed. The experiment was repeated twice using five animals with similar results. The control cell line, H460, formed tumors within the measured time. However, retinoic acid-treated H460 cells formed tumors grew into much larger in size compared to those fromuntreated cells (Fig. 4). Thus, we can correlate the tumor formation enhancement with overexpression of EGF-R on H460 cell surface.

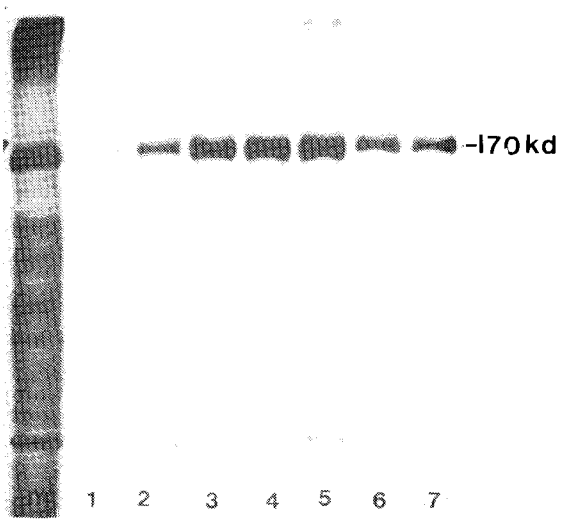


Fig.3: Metabolic labeling with ^{35}S-methionine. H460 cells were treated with retinoic acid for different time period. The media were removed and replaced with methionine-free medium. The cells were labeled with ^{35}S-methionine for 1 h. Following RIPA buffer treatment, the clear lysates were immunoprecipitated with R_1 anibody. Equal amounts of radioactive materials were resolved with 7.5% SDS-PAGE gels. Lanes 1, 2, 3, 4, 5 and 6 represent retinoic acid treatment for 0, 16, 24, 32, 48, and 72 h, respectively. Lane 7 represents cells grown for 24h in retinoic acid-free medium after 72 treatment with retinoic acid. Lane m represents A431 cells as control.

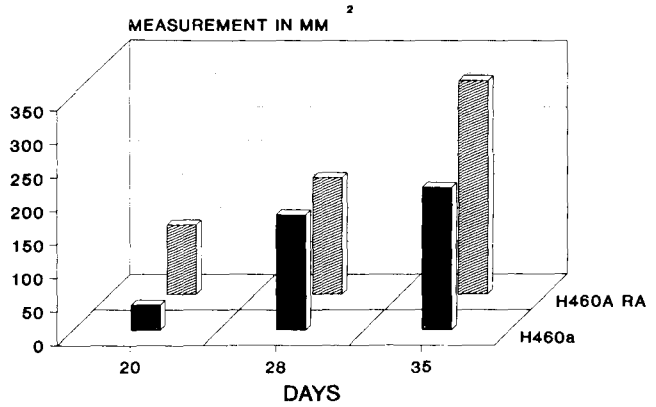


Fig.4: Tumorigenicity in nude mice. Retinoic acid-pretreated cells (2×10^5 cells) were inoculated subcutaneously in 6-week-old female *nu/nu* mice. The cross-sectional diameters of the tumors were measured externally at different time points. Tumor volume was calculated according to Mukhopadhyay *et al.* (1991). The symbols reflect mean value of two experiments with five mice each.

Discussion

Overexpression of EGF-R has always been correlated with the formation and progression of tumors (Chaffanet *et al.*, 1992). The EGF-R related mitogenic activity is associated with TGF-α-mediated autocrine regulation (Salomon *et al.*, 1990). Retinoic acid is known to induce ^{125}I-labeled EGF binding capacity in a number of cell lines (Oberg and Carpenter, 1989). In pulse-chase experiment of this cell line, it is shown that the stability of EGF-R increased after retinoic acid treatment (Oberg and Carpenter, 1989). In this study, we have shown that human lung cancer cells express elevated levels of EGF-R when treated with retinoic acid. The protein expression level reached at the maximal level at 48 h and declined thereafter. However, upregulation of H460 EGF-R expression did not show significant increase of tritiated thymidine uptake of retinoic acid-treated H460 cells when stimulated with exogenous EGF (unpublished data). Treatment of H460 with retinoic acid has been reported to induce the formation of retinoic acid receptors (Jetten, 1980). This work implies that, in human tumor cell line H460, a rapid transient formation of EGF-R is the indirect result of retinoic acid-induced EGF-R upregulation. The accumulation of EGF-R could be the steady rate EGF-R mRNA synthesis. Further run-on transcription experiment on H460 mRNA rate formation should clarify if elevated EGF-R level is due to the transcriptional escalation that is reflected at protein level.The enhanced phosphorylation activities of EGF-R corresponds with transiently elevated expression of protein levels. This phenomenon is distinct from reported kinase activity down-regulation due to the suppression of glycosylation of EGF-R in cultured human head and neck squmous carcinoma cells by retinoic acid (Kim *et al.*, 1992). The stability of EGF-R synthesis was manifested after 72 h treatment and maintained thereafter as indicated in retinoic acid-free media after 72 h treatment. The stability could also involve induction of some factors that inhibit protease activity. Otherwise, the intracellular retinoic acid may continue exerting EGF-R expression in drug-free medium. Retinoic acid-treated cells formed tumors with larger volumes. Thus, this work clearly demonstrated that EGF-R overexpression is closely associated with tumor progression. Earlier report indicates that H460 cells express mutated retinoic acid receptor, thus retaining resistance toward retinoic acid treatment (Gerdts *et al.*, 1993). This work further indiates that the abnormal retinoic acid receptor and EGF-R expression regulation is closely associated with tumor progression.

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維生素甲酸對人體肺癌細胞株上皮生長因子受體表現具調節作用

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摘 要

維生素甲酸有調節細胞訊息調節作用。人類肺癌細胞株H460,於維生素甲酸培養下,上皮生長因子受體有增生現像。上皮生長因子受體的表現隨時間而逐漸增加,至48小時為最高,而後開始下降。這種現像可由碘^{125}標示上皮生長因子結合變化,上皮生長因子受體磷酸化變異,及硫^{35}活體標示得知。若將維生素甲酸移去,則增生上皮生長因子受體會維持穩定表現。而所誘導增生上皮生長因子受體也使裸鼠腫瘤體積增加,也間接證實上皮生長因子受體與腫瘤形成的確有密切關係。這也是首次證明維生素甲酸與蛋白質合成及代謝速率調節有關。

關鍵詞：人類肺癌細胞、上表生長因子受體、維生素甲酸