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Inhibition of Epidermal Growth Factor Receptor Activity by Retinoic Acid in Glioma Cells

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The growth inhibitory effects of exogenously added retinoic acid (RA) on various cultured human glioma cells was observed to be heterogenous, with an ID_{50} ranging from 10^{-7} M to no response. The protein tyrosine kinase activity of epidermal growth factor receptor (EGF-receptor) appeared to parallel the cell's growth responsiveness to RA. Cells sensitive to RA-induced growth inhibition exhibited a dose-dependent decrease in EGF-receptor activity, whereas RA-resistant cells showed no alterations in EGF-receptor protein tyrosine kinase activity or expression. The modulation of EGF-receptor by RA was further examined with RA-sensitive (LG) and -resistant (NG-1) cell lines. Both cell lines were approximately equal in their ability to bind and internalize epidermal growth factor in the presence or absence of RA. Several independent assays suggested that the inhibition of EGF-receptor activity was independent of protein kinase C modulation as mediated by phorbol myristate acetate. However, alterations in associated glycoconjugates of EGF-receptor were observed among the sensitive cells but not the resistant cells. These results suggest RA-induced growth inhibition in sensitive cells may arise, at least in part, through alterations in EGF-receptor activity and structure.

Key words: growth regulation, EGF-receptor, signal transduction, glycoconjugates, tyrosine kinase

The overexpression of epidermal growth factor receptor (EGF-receptor) has been observed in a number of human cancers [1–4], particularly glioblastomas, in which amplification of EGF-receptor gene or proteins products has been observed in about 50% of the tumors examined [5–7]. A possible role of EGF-receptor in cellular transformation and oncogenesis was initially suggested by Downward et al. [8], who revealed sequence homology between EGF-receptor and the v-*erb*-B oncogene protein product [9]. Subsequently, altered EGF-receptor genes and protein products were found in gliomas [6,10–12]. However, further studies revealed that overexpression of EGF-receptor itself was insufficient to cause transformation [13,14]. Cellular transformation required the presence of EGF-receptor's ligands: epidermal growth factor (EGF) [15] or transforming growth factor-alpha (TGF-alpha) [14]. Both ligands have been shown to induce the protein tyrosine kinase activity and autophosphorylation of EGF-receptor as

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well as DNA synthesis. These findings suggest that EGF-receptor and its regulatory mechanism(s) may represent plausible targets for tumorigenesis and therapeutic modulation.

The regulation of ligand binding (affinity) to EGF-receptor and the tyrosine kinase activity of EGF-receptor have been the focus of a number of studies. Three non-mutually exclusive pathways have been generally elucidated: a) the phosphorylation of the threonine 654 residue of EGF-receptor by protein kinase C (PKC) [16–19]; b) the dimerization of EGF-receptor [20–22]; and c) the effects of membrane gangliosides [23–25]. Several studies have demonstrated that the phosphorylation of the threonine 654 residue of EGF-receptor by PKC results in an inhibition of high-affinity binding of EGF to EGF-receptor and a decrease in tyrosine protein kinase activity [16–19]. The activation of PKC by phorbol 12-myristate 13-acetate (PMA) also may result in internalization of unoccupied EGF-receptor, yielding an alteration in receptor number [26]. However, recent investigations have implicated another independent mechanism for regulation of EGF binding affinity; this mechanism is currently unknown but appears to consist of a rapid transient phase and a slower permanent phase [27,28]. Also, Bjorge and Kudlow [29] reported that EGF-induced growth inhibition of the A431 cell line is independent of PKC activity and down-regulation. Therefore, although PKC has been intimately implicated in the regulation of EGF-receptor activities, particularly modulation of protein tyrosine kinase, alternative regulatory mechanisms for EGF-receptor may exist.

Our previous investigations have shown an apparent correlation between retinoic acid (RA)-induced growth inhibition and a dose-dependent decrease in the protein tyrosine kinase activity of EGF-receptor in certain sensitive human glioma cells [30]. This present study was undertaken to examine the possible biochemical mechanism(s) of RA-induced modulation of EGF-receptor, and particularly the plausible role of PKC.

MATERIALS AND METHODS

Cells and Cell Growth

The human glioma cells were obtained, characterized, and cultured as previously described [30,31]. For RA treatment, cells were grown for 3 days in growth medium (1:1 mixture of Dulbecco's modified minimal essential medium and Ham's F-12 medium) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), and containing the appropriate dilution of dimethyl sulfoxide (DMSO) in the presence or absence of RA (5 μM, unless otherwise stated; RA stock solution was 10^{-2} M in DMSO). Cell numbers were determined after trypsinization using a Coulter cell counter or a hematocytometer [30].

Epidermal Growth Factor Receptor Expression

The binding of ^{125}I-labeled epidermal growth factor (EGF; receptor grade, Collaborative Research, Boston, MA) to the various cells after treatments with RA, PMA, or both was performed as previously described [30,31]. Quantitation of the binding was analyzed by the method of Scatchard [32]. The specific activity of EGF ranged from 8.5 to 6.3 ($\times 10^5$) cpm/pmol and binding was performed in 6 well multiwell dishes and in 1 ml of binding buffer [31]. For treatment with PMA, cells were exposed to 10 μM PMA for 25 min at 37°C prior to initiation of the analyses [17,18]. Internalization of bound

^{125}I-labeled EGF was examined by the procedure described by Haigler et al. [33]. Briefly, the various cell cultures were incubated with ^{125}I-labeled EGF for 45 min at 4°C, washed three times in binding buffer (phosphate-buffered saline (PBS) containing 0.05% bovine serum albumin), and followed by warming of the cells to 37°C. After various times, individual cultures were washed three times with ice-cold binding buffer, treated with 0.2 M acetic acid containing 0.5 M NaCl for 6 min at 4°C, and then cells were lysed with 1% sodium dodecyl sulfate (SDS) in 50 mM NaOH. The percentage of internalized EGF was determined from the difference between total cell-associated radioactivity and cell surface-associated radioactivity [33].

Immunoprecipitations

The activity of EGF-receptor was determined by autophosphorylation of the receptor in immuno-complex-kinase assays or phosphorylation of exogenously added angiotensin II, based on equal cell numbers, as previously described [30]. The phosphorylation status of EGF-receptor after exposure to the different agents (no treatment, PMA, RA, EGF, or a combination thereof) was examined by immunoprecipitation from cells metabolically radioactively labeled with $^{32}P_i$ (1 mCi/ml) in phosphate-free medium containing 2% dialyzed fetal bovine serum. The cells were washed twice with ice-cold PBS, followed by solubilization with 1 ml of 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, 20 mM sodium phosphate, 2 mM sodium pyrophosphate, 150 mM NaCl, 5 mM EDTA, 20 mM NaF, 1 mM $NaVO_3$, 1% aprotinin, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 4 mM iodoacetic acid, and 20 mM HEPES, pH 7.4. The extracts were further homogenized with a Dounce homogenizer (20 strokes), and then centrifuged at 100,000*g* for 1 h at 4°C. The lysates were adjusted to yield equal cell numbers, after which anti-EGF receptor antibody (R1; Amersham, Arlington Heights, IL) was added and the lysates incubated for 1 h with shaking at 4°C. The immunocomplexes were precipitated with Pansorbin (30 μl; Calbiochem, La Jolla, CA) for 30 min. The precipitates were washed and subjected to polyacrylamide gel electrophoresis in SDS as previously described [30,31]. The gels were then dried and exposed to X-ray film.

The phosphoamino acid analysis was performed as previously described [34]. Briefly, the dried gel corresponding to EGF-receptor was rehydrated in 50 mM NH_4HCO_3, washed, and then incubated overnight in 3 ml of NH_4HCO_3 in the presence of 100 μg/ml TPCK-treated trypsin [18]. After the addition of another aliquot of trypsin (50 μg/ml) and an additional 8 h incubation, the supernatant was lyophilized. The tryptic digest was then hydrolyzed in 6 N HCl for 1 h at 110°C, followed by removal of the acid. The phosphoamino acids were then separated on a SynChroPak AX 300 high-performance liquid chromatography anion exchange column (25 × 0.4 cm) eluted with 15 mM K_2HPO_4 at pH 3.8 [34].

To examine the glycoconjugates associated with EGF-receptor, cultures were treated with or without RA for 3 days, the cells lysed, and EGF-receptor immunoprecipitated as described above. The immuno-complex was divided into equal aliquots and subjected to polyacrylamide gel electrophoresis, followed by transfer of the proteins to nitrocellulose paper (0.1 μm) overnight at 30 V [31]. The nitrocellulose papers were then incubated for 2 h in PBS containing 3% BSA, followed either by addition of anti-EGF-receptor monoclonal antibody (ICN Biochemicals, Lisle, IL) or biotinylated lectins (Vector Labs., Burlingame, CA). The blots were then incubated for 1 h at room

temperature, followed by three washes with TTBS (50 mM Tris HCl, pH 7.2; 100 mM NaCl containing 0.05% Tween 20). The anti-EGF receptor immunoblots were then incubated with ^{125}I-labeled anti-mouse IgG, while the lectin blots were incubated with ^{125}I-labeled avidin for 1 h, followed by an additional three washes with TTBS. The blots were dried, exposed to X-ray film, and then densitometric scans of the films were performed or the labeled bands were excised from the gel and the associated radioactivity quantitated [35]. Alternatively, the cells were metabolically radioactively labeled with either [^{3}H]glucosamine, [^{3}H]galactose, [^{3}H]mannose, [^{3}H]fucose (40 μCi/ml), or [^{35}S]methionine (20 μCi/ml) overnight, and then subjected to immunoprecipitation and separation of EGF-receptor as described above.

RESULTS

The growth-inhibitory effects of RA on various cultured human glioma cells were examined. Four of eight cell lines exhibited at least some degree of decreased cellular growth in the presence of RA (5 μM), as shown in Figure 1A. Additionally, the growth inhibition was observed to be dependent on the concentration of exogenously added RA in the sensitive cell lines (Fig. 1B). The EGF-receptor's protein tyrosine kinase activity of the various cells also showed a decline, as evidenced by the autophosphorylation of the receptor, which paralleled the growth inhibition (Fig. 1A). Exposure of the cells to RA for at least 48 h was necessary before modulation of growth or kinase activity could be observed. RA added directly to the cellular lysates had no effect on EGF-receptor's protein tyrosine kinase activity. The inhibition of EGF-receptor activities also appeared to be relatively specific to the action of RA, as other biological agents that caused growth inhibition such as beta-interferon showed no modulation of EGF-receptor activity. To examine the effects of RA in greater detail, two cell lines were chosen for further analysis: LG cells which are RA sensitive and NG-1 cells which are RA insensitive. The dose-dependent inhibition of growth and EGF-receptor activity, as measured by autophosphorylation of the receptor or exogenous substrate phosphorylation (angiotensin II), of the two cell lines is shown in Figure 1B. Additionally, the inhibition of EGF-receptor autophosphorylation activity induced by RA is observed regardless of LG cell density.

The phosphorylation of the threonine 654 residue of EGF-receptor by PKC has been shown to influence the activity of EGF-receptor [17–19]. To examine whether RA acted through activation of PKC, several series of studies were performed. The binding of ^{125}I-labeled EGF to LG and NG-1 cells was studied after treatment of the cells with RA, PMA, or both (Fig. 2). Exposure of LG cells to RA or PMA resulted in approximately equal effects, whereas the combination of the two agents appeared to exhibit an additive effect. Quantitative Scatchard analysis of the data obtained from the binding curves for LG cells indicated that the number of EGF-receptors was not altered, but the affinity of EGF binding was modulated to about the same extent by either RA or PMA, with about a fourfold change in K_d (Table I). The action of both agents was additive, with about a tenfold change. In contrast, treatment of NG-1 cells with either agent individually resulted in only minor effects, with PMA being slightly more potent (Fig. 2). The combination of RA and PMA appeared to be synergetic, with a twofold change in K_d and a threefold decrease in receptor number (Table I). However, it should be emphasized that only the alterations in binding induced by both RA and PMA (a tenfold change in K_d for LG cells and a threefold change in receptor number for NG-1

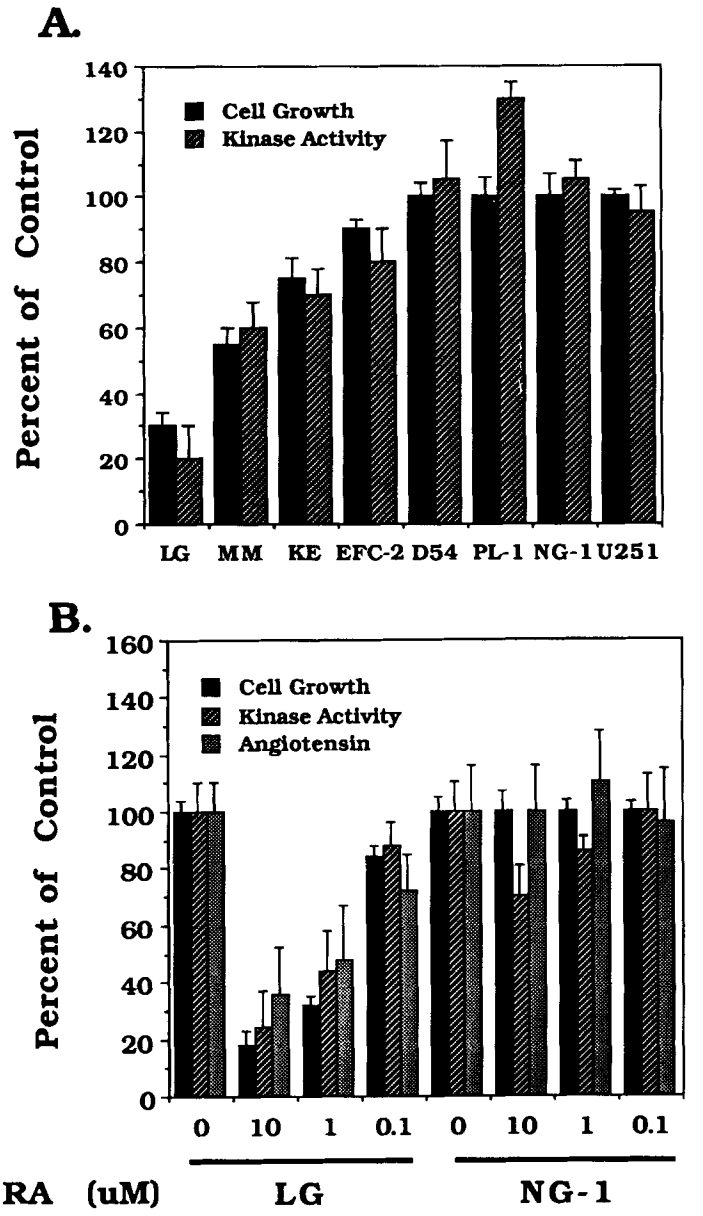

Fig. 1. Comparison of growth-inhibitory and EGF-receptor tyrosine kinase activity in response to exogenously added RA. **A:** The growth inhibition and autophosphorylation kinase activity of RA-treated (5 μM) and untreated glioma cell lines. Control (100%) represents the appropriate various untreated cell's growth and autophosphorylation activity. **B:** The growth inhibition, phosphorylation activity of EGF-receptor as assessed by autophosphorylation or phosphorylation of exogenous substrate (angiotensin II) in immunocomplex-kinase assays of LG and NG-1 cells after 3-day exposure to various concentrations of RA. The standard deviations are denoted by the error bars. Similar results were obtained in two independent experiments.

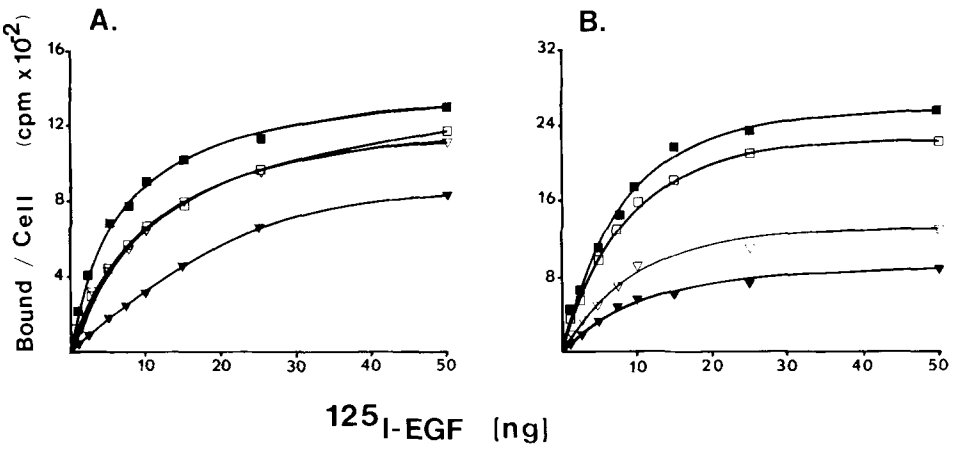

Fig. 2. The dose-saturation binding curves for specific binding of ^{125}I-labeled EGF to LG (**A**) and NG-1 (**B**) cells. The binding was performed at 4°C after no treatment (■) or exposure to RA (□), PMA (▽), or both (▼). The treatment times were 3 days for RA (5 μM) and 25 min for PMA (10 μM), both at 37°C. The nonspecific binding was determined with a 100-fold molar excess of unlabeled EGF. Scatchard analysis [32] of the binding data yielded linear plots, which were analyzed by linear regression analyses, shown in Table I. Similar results were obtained from three independent experiments, and the standard deviation of the individual measurements are within the symbol size.

TABLE I. Effects of RA and PMA on ^{125}I-Labeled EGF Binding to Glioma Cells*

Cell line	Treatment	Receptor No./ cell ($\times 10^{-4}$)	K_d (M) ($\times 10^{-9}$)
LG	Control	8.6	0.8
	RA	10.3	3.1
	PMA	9.4	2.1
	RA + PMA	9.7	7.9
NG-1	Control	22.9	2.2
	RA	18.3	2.3
	PMA	16.8	3.1
	RA + PMA	7.0	5.2

*The cells were grown in the presence or absence of 5×10^{-6} M RA for 72 h. PMA (10 μM) was added to the appropriate cultures 25 min before initiation of binding of ^{125}I-EGF to the cells as described in Materials and Methods. The results were analyzed by the method of Scatchard [32], to calculate the No. of sites per cell and the K_d. The *P* values for the binding constants of the individual agents were <0.05 and <0.005 for the combination of agents as compared to control. The nonspecific binding was performed in the presence of a 100-fold excess of cold EGF averaged less than 3% of total binding. The cells exhibited significant amounts only of low-affinity binding.

cells) would represent potentially significant biological modulations, although most of the other observed changes are statistically significant.

To further determine the effects of RA and PMA on EGF-receptor activities, we examined the internalization of ^{125}I-labeled EGF in LG and NG-1 cells after they were exposed to the various agents. Treatment of either cell line with RA produced no significant changes in the rate or maximum amount of internalization of the labeled ligand at 37°C compared with untreated control cells (Fig. 3). The control and RA-treated LG and NG-1 cells both internalized 50% of the bound ligand by approximately

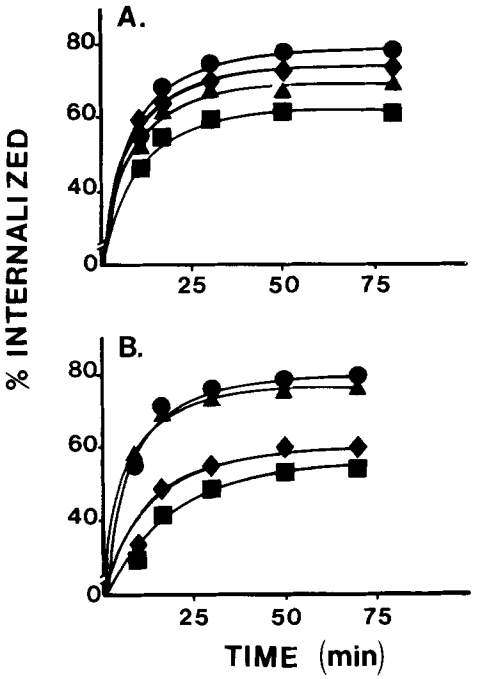

Fig. 3. The internalization of ^{125}I-labeled EGF by LG (A) and NG-1 cells (B) after no treatment (●) or exposure to RA (▲); PMA (♦); or both (■). Labeled EGF was bound to the cells for 45 min at 4°C; the cells were then washed to remove unbound ligand and warmed to 37°C. After various times of incubation, the cells were washed with acid to determine cell surface-associated ligand and then lysed with 1% SDS in 50 mM NaOH to calculate total bound ligand. The difference between total bound and cell surface-associated ligand is the % internalized [33]. The results are given as the mean of triplicate wells, and the standard error is within the size range of the symbols used. The results were similar in duplicate independent experiments.

8 min ($t_{1/2}$ = 8 min). Prior exposure of either cell line to PMA showed only a slight decrease in the rate and extent of internalization for LG cells ($t_{1/2}$ = 9 min) and a larger decrease for NG-1 cells ($t_{1/2}$ = 26 min). The combination treatment with the two agents again appeared to have a synergetic effect ($t_{1/2}$ = 20 min for LG cells and 50 min for NG-1 cells). The combination treatment also decreased the maximum ligand internalization from about 78% to 58% in LG cells and from 77% to 52% in NG-1 cells at 75 min. The NG-1 cells were also more sensitive to the effects of PMA on both the binding and internalization of EGF than the RA-sensitive LG cells.

The phosphorylation status of the EGF-receptor in LG cells metabolically radioactively labeled with inorganic phosphate was examined following RA and PMA treatments. The incorporation of phosphate into EGF-receptor was approximately equal in the presence or absence of RA at the basal level (no treatment) (Table II). Treatment of the cells with PMA resulted about a twofold increase in phosphate incorporation over that of the basal level, and again RA had no effect. Furthermore, RA treatment did not alter the composition of phosphate incorporation into the amino acids of EGF-receptor with or without PMA exposure (Table II). The addition of EGF resulted in approximately a 2½-fold increase in phosphate incorporation, which was predominantly on tyrosine residues. However, RA treatment of the LG cells decreased the phosphorylation

TABLE II. Phosphoamino Acid Composition of EGF-Receptor From LG Cells After Treatments With RA, PMA, and EGF

Treatment[a]	Phosphothreonine (%)	Phosphoserine (%)	Phosphotyrosine (%)	Total CPM[b]
Control	23	47	30	1,250
Control + RA	22	47	32	1,380
PMA	37	40	23	3,400
PMA + RA	37	42	21	3,200
EGF	19	18	63	3,950
EGF + RA	33	35	31	1,840

[a]LG cells were treated wtih RA for 3 days, and PMA (10 μM) or EGF (100 ng/ml) for 25 min. The cells were metabolically radiolabeled with [^{32}P]phosphate (1 mCi/ml) overnight before treatment with PMA or EGF in the presence or absence of RA.

[b]After acid hydrolysis, the phosphoamino acids were separated by a HPLC column equalibrated and eluted with 15 mM K_2PO_4, pH 3.8, at 2 ml/min on a Waters HPLC. The elution positions of the phosphoamino acids were determined using unlabeled standards. The quantitation of the individual peaks was accomplished by liquid scintillation spectrophotometry.

of tyrosine residues of EGF-R by approximately 50% upon addition of EGF (Table II). The decrease in phospho-tyrosine residues of RA-treated EGF-receptor and inhibition of exogenous substrate phosphorylation (Fig. 1B) suggests RA is acting through inhibiting the kinase activity of the receptor.

The combined results of the previous experiments also suggested that RA was not acting through activation of a PKC-mediated mechanism. Therefore, investigations were undertaken to examine the possible structural modulations of EGF-receptor that might be responsible for RA-mediated inhibition of the tyrosine kinase activity. No alterations were observed in partial proteolytic analyses between EGF-receptor isolated from LG cells treated with or without RA and metabolically labeled with [^{32}P]phosphate or [^{35}S]methionine. However, decreases in the incorporation of ^{3}H-labeled glucosamine (45%), fucose (10%), and mannose (20%) into EGF-receptor was observed in RA treated cells as compared to untreated cells, based on equal incorporation of [^{35}S]methionine into EGF-receptor. A 230% increase was observed in the incorporation of galactose into the EGF-receptor from RA-treated cells compared with control cell, even though decreased synthesis of EGF-receptor itself was observed (20–40% decrease in [^{35}S]methionine incorporation). Since the alteration in incorporation of the metabolic precursor may be the result of altered pool sizes or specific activity of incorporation, studies of lectin binding to equal quantities of isolated EGF-receptor were performed. EGF-receptor was immunoprecipitated from LG and NG-1 cells that were treated with or without RA, separated by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose paper. One paper from each identical series of gels was then incubated with anti-EGF-receptor monoclonal antibody to determine equal quantities of EGF-receptor. Alternatively, the other filter papers were incubated with biotinylated lectins, iodinated avidin and then exposed to X-ray film. A significant decrease in the binding of wheat germ agglutinin, *Phaseolus vulgaris-E* aggutinin, *Lens culinaris* agglutinin, and *Ricinus communis* agglutinin-I was found between the RA untreated and treated EGF-receptors from LG cells but not NG-1 cells (Table III). A number of other lectins showed no differences or no significant binding to the various isolated EGF-receptors from either cell line (Table III).

TABLE III. Analysis of Binding of Lectins to Immunoprecipitated EGF-Receptor Isolated From Cells Treated or Untreated With Retinoic Acid

Lectin	LG(RA)/LG(C)[a]	NG-1(RA)/NG-1(C)	Intensity[b]	Specificity[c]
Wheat germ agglutinin	0.6	0.9	H	GlcNAc
Lens culinaris agglutinin	0.6	1.1	L	Man
Phaseolus vulgaris agglutinin-E	0.6	1.1	M	Gal-GlcNAc
Ricinus communis agglutinin I	0.7	1.1	M	Gal
Concanavalin A	1.0	1.0	M	Man
Phaseolus vulgaris agglutinin-L	1.0	1.1	L	GlcNAc
Dolichos biforus agglutinin	NB	NB[d]	—	GalNAc
Ulex europaeus agglutinin	NB	NB	—	Fuc
Peanut agglutinin	NB	NB	—	Gal-GalNAc
Sophora japonica agglutinin	NB	NB	—	Gal,GalNAc
Bandeierea simplicifolia lectin	NB	NB	—	Gal,GalNAc

[a]EGF-receptor was immunoprecipitated from the various cells as described in Materials and Methods, and the immuno-complex was divided into four equal aliquots for separation by SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose paper. Each paper contained EGF-receptor from all the cell lines. One blot of each set was incubated with anti-EGF-receptor monoclonal antibody, while the other three were incubated with biotinylated lectins, and then the blots were incubated with appropriate ^{125}I-labeled secondary agent. After exposure of X-ray film to the blots, the film was subjected to densiometric analysis. The resultant quotient is the quantitation of lectin binding to EGF-receptor from RA-treated cells compared to untreated cells, based on equal amounts of EGF-receptor.

[b]Intensity is a relative estimation of lectin binding as determined by the required exposure time of the blot to the X-ray film; H = high, less than 12 h; M = medium, 24 h; L = low, 48 h.

[c]Simple sugar specificity for inhibition of lecting binding as defined [49]. A 0.2 M solution of the saccharides with the lectin during the incubation was used to determine specificity of lectin binding. GlcNAc, N-acetylglucosamine; Gal, galactose; Man, mannose; GalNAc, N-acetylgalactosamine, Fuc, fucose.

[d]No binding.

DISCUSSION

Previously, we demonstrated a dose-dependent growth inhibition induced by RA in certain cultured human glioma cells that apparently correlates with a decrease in the EGF-receptor tyrosine kinase activity of those cells [30]. Several investigators have reported on studies implicating a PMA-mediated PKC activation resulting in inhibition of EGF-receptor protein tyrosine kinase activity via phosphorylation of threonine 654 (16–19,36). This investigation was designed to examine possible biochemical mechanisms responsible for the RA-mediated inhibition of EGF-receptor activity, especially the possible mediation by PKC.

PMA-mediated activation of PKC has been shown to modulate the activities of EGF-receptor in situ and in vitro [36,37]. These activities include a decrease in receptor affinity for EGF-receptor ligands as well as lowering the protein tyrosine kinase activity. PMA has also been implicated in inducing internalization of unoccupied EGF-receptor

[26], which appears to occur with NG-1 cells but not LG cells in this study. Recently, the pivotal role of PKC as the regulator of EGF-receptor has been questioned, although phosphorylation of threonine 654 still appears to play a major role, particularly for the protein tyrosine kinase activity of the receptor [19,27]. In this investigation, the effects of RA and PMA appeared to be additive in their actions, even though both agents were used at relatively high concentrations. Treatment of the cells with either RA or PMA revealed a slight but consistent increase in the K_d of EGF-receptor. Other investigators have reported an increase or decrease of the number of receptors upon treatment with RA depending on the cell type examined [38,39]. The internalization of bound ^{125}I-labeled EGF also exhibited a similar synergic response to RA and PMA treatment. Furthermore, in both assays the effects of PMA were equal to or greater than the effects of RA. The results from these studies suggest that RA and PMA act through independent mechanisms.

The addition of PMA to various cells has been shown to increase the phosphorylation of a number of amino acid residues of EGF-receptor, although only the phosphorylation of residue threonine 654 appears to correlate with the observed decrease in the receptor's affinity for EGF and protein tyrosine kinase activity [36]. Direct evidence was provided by site-directed mutagenesis of threonine 654 to alanine of EGF-R [26]. The treatment of LG cells with RA did not alter the basal level of phosphorylation of EGF-receptor or the ability of PMA to stimulate phosphorylation of the receptor. Also, the composition of the phosphorylated amino acids of EGF-receptor was not changed in the basal or PMA-stimulated receptor in the presence or absence of RA. The relatively low number of EGF-receptors expressed by LG cells makes the analysis of the phosphorylation status of theonine 654 peptides difficult; however, preliminary evidence suggests that RA treatment does not affect its ability to be phosphorylated by PMA-mediated activation of PKC. The combination of these results and the binding data suggest that RA modulation of EGF-receptor activity is not mediated through a PKC-like activity.

An alteration in the synthesis and expression of the carbohydrate moieties associated with EGF-receptor was shown in LG cells but not in the RA-resistant NG-1 cells. Recently, a difference in EGF-receptor protein tyrosine kinase activity and a reduction in the number of high-affinity receptor sites were described among variant clones of A431 cells expressing or lacking blood group A antigen on the cells and on EGF-receptor itself [40]. The cells expressing the A antigen were shown to have decreased kinase activity per receptor. Such a modulation would agree with our observation of increased galactose incorporation into RA-treated LG cells, that the N-acetyl-D-galactosamine-galactose residue is part of the structural determinant of blood group A antigen. Also, Cummings and co-workers [41] have reported that N-acetylgalactosamine residues occur predominantly at the nonreducing termini of EGF-receptor oligosaccharides. However, we failed to observed any significant amounts of binding of *Dolichos biforus* agglutinin (DBA) to EGF-receptor from either cell line, with or without RA treatment. Also, *Ulex europaeus* lectin was not observed to bind to the various EGF-receptors in this study. The EGF-receptors and the variant A431 cells could be easily distinguished by these two lectins [40]. This raises several possibilities: first, only certain and not all of the glycoconjugates associated with EGF-receptor may play a role in modulation of the receptor activity. Alternatively, the majority of studies directed at oligosaccharides of EGF-receptor have been performed using A431 cells which exhibit blood group A specificity [42], and other cells may express altered antigenic specificity. Also, the

antigen may be expressed at minute amounts on the EGF-receptors from LG and NG-1 cells, below our detection methods; however, we can easily discern the binding of a number of other lectins, making this an unlikely possibility. Additionally, the decreased incorporation of fucose, mannose, and particularly glucosamine into EGF-R of cells treated with RA, accompanied by the observed decreased binding of *Phaseolus vulgaris* agglutinin-E, wheat germ agglutinin, and to EGF-receptor from the RA-treated cells, suggests other alterations in glycoconjugate synthesis and expression are probably occurring. Furthermore, although both of these studies implicate alterations of associated glycoconjugates of EGF-receptor correlating the modulation of receptor function, a structure-function relationship has not been established. Another possibility is that the alterations in carbohydrate synthesis in these cells are also effecting the expression of glycolipids which may then modulate EGF-receptor activities [23–25]. The exact roles of the altered glycoconjugates observed in these studies still remains to be clearly defined.

Previous studies have shown that the addition of carbohydrate to EGF-receptor appeared to represent a necessary step for the activation of EGF-receptor; however, once activated, the glycoconjugates could be removed without apparent loss of function [43]. Also, incomplete glycoconjugate synthesis (inhibition by swainsonine or monensin) yielded fully functional EGF-receptors [44]. Similarily the insulin and insulin-like growth factor II receptors have been reported to require posttranslational activation for ligand binding [45,46], although incomplete processing of receptors mediated by swainsonine again yields functional receptors [47]. The modulation of glycoconjugates has been shown to accompany many biological processes, including cellular transformation and tumor progression [48]; however the potential role(s), if any, of glycoconjugate modulation in growth regulation remain to be elucidated.

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