Action of Epidermal Growth Factor and Retinoids on Anchorage-dependent and -independent Growth of Nontransformed Rat Kidney Cells

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ABSTRACT

Treatment of two nontransformed rat kidney cell lines with either retinoic acid or epidermal growth factor enhances anchorage-dependent cell growth and reduces cell substratum adhesiveness significantly, whereas the cell morphology is only moderately affected. Simultaneous treatment of these cells with these factors results in a dramatic change in cell morphology and increases cell growth and reduces cell substratum adhesiveness even further.

Treatment of nontransformed rat kidney cells with certain retinoids enhances the binding of 125I-labeled mouse epidermal growth factor 2- to 3-fold. Addition of epidermal growth factor stimulates anchorage-independent growth of these cells. Retinoids enhance colony-forming ability in soft agar produced by epidermal growth factor, although retinoids by themselves do not affect anchorage-independent growth. Growth in soft agar induced by epidermal growth factor or epidermal growth factor plus retinoic acid treatments appears to be a reversible trait. Addition of epidermal growth factor stimulates secretion of plasminogen activator, whereas this production is not influenced by retinoids. A transformed rat kidney cell line, which exhibits very low epidermal growth factor binding and grows progressively in soft agar, is not significantly affected by retinoid treatment.

INTRODUCTION

EGF3 is a polypeptide that binds to specific receptors at the cell surface of responding cells (4, 6). Binding of EGF to its receptor triggers a cascade of events such as phosphorylation of the EGF receptor and maybe other substrates (7), aggregation of the EGF-receptor complexes into coated pits, followed by internalization and degradation of EGF in the lysosomes (5, 15, 23). One or more of the steps in this cascade must ultimately lead to the many biological and biochemical responses induced by EGF treatment, such as stimulation of mitogenesis and increased transport of glucose and amino acids (4, 6). Binding of EGF is affected by a large number of conditions such as transfection (10, 36) and treatment with phorbol esters (24, 35) of the EGF receptor and maybe other substrates (7), aggregation of the EGF-receptor complexes into coated pits, followed by internalization and degradation of EGF in the lysosomes (5, 15, 23). One or more of the steps in this cascade must ultimately lead to the many biological and biochemical responses induced by EGF treatment, such as stimulation of mitogenesis and increased transport of glucose and amino acids (4, 6). Binding of EGF is affected by a large number of conditions such as transfection (10, 36) and treatment with phorbol esters (24, 35) and glucocorticoids (1, 30) some of which reduce, whereas others enhance, EGF binding. We have shown recently (19) that analogues of vitamin A (retinoids) and especially retinoic acid can enhance EGF binding to a large number of cell lines of various cell types, probably due to an increase in the number of available receptor sites (18, 20). It has been demonstrated that this group of compounds can modulate the growth of certain cell types in vivo as well as of a large number of established cell lines (19, 22, 28). In certain instances, retinoids have a stimulating effect whereas, in others, they inhibit cell growth (11, 19, 21, 34).

Anchorage-independent growth has been shown to be an in vitro property that often accompanies the malignant phenotype of cells (2, 8). One of the factors that has been suggested to be involved in this process is the production of plasminogen activator (17, 31). In this paper, we report the influence of EGF and retinoic acid on anchorage-dependent and -independent growth of NRK cells showing synergism between the action of these 2 compounds and discuss this in the light of the effects on EGF binding and plasminogen activator production.

MATERIALS AND METHODS

Cell Culture. Normal rat kidney fibroblast cells NRK 536-3(SA6) were kindly provided by Dr. J. De Larco (National Cancer Institute, Frederick, Md.). At the 30th passage, these cells were recloned, and several spontaneously transformed and normal cell lines were obtained. Clone 536-3-1 and clone 536-3-14, which show strong contact inhibition, and clone 536-3-9, which consists of spontaneously transformed cells, were used in our studies. Cells were grown in DMEM containing 10% fetal calf serum, penicillin, and kanamycin (100 units/ml each) and streptomycin (100 μg/ml). All components of this medium were obtained from Grand Island Biological Co., Grand Island, N. Y. Cells were treated with retinoids at 10–8 M; the action of retinoic acid on several parameters appeared to be optimal at this concentration. Stock solutions of retinoids (6.8 × 10–3 M) were made in ethanol; control experiments were carried out containing ethanol only.

Anchorage-dependent Cell Growth. The effects of retinoic acid and EGF on anchorage-dependent cell growth were determined in duplicate by measuring cell number or [3H]thymidine incorporation as described previously (21).

Cell Detachment Assay. Cell substratum adhesiveness was determined in duplicate dishes as described previously (22) by measuring the number of cells that are detached and remain attached after treatment with trypsin (100 μg of trypsin per ml of phosphate-buffered saline [contants (g/liter): KCl, 0.2; NaCl, 8.0; KH2PO4, 0.2; Na2HPO4·7H2O, 113.8; and glucose, 9.5 x 10−3 dpm/ml] in binding medium (DMEM) containing bovine serum albumin (1 mg/ml) and 50 mw A2/A2 (bis[2 hydroxyethyl]-2-amino-ethanesulfonic acid (pH 6.8), followed by 3 consecutive washings with 3 ml of binding medium. Cells were solubilized in triy buffer (0.1 M Tris-HCl, pH 7.4) containing 0.5% sodium dodecyl sulfate and 1% EDTA. Nonspecific binding was determined in the presence of 10 μg of unlabeled mouse EGF (Collaborative Research) and amounted to less than 3% of total label bound.

Anchorage-independent Growth. Trypsinized cells were added to

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complete medium supplemented with 0.3% agar (Difco Laboratories, Inc., Detroit, Mich.). Aliquots of 2 ml were pipetted onto a base layer of 3 ml of complete medium plus 0.5% agar in 60-mm Petri dishes (Falcon). Experiments were carried out in duplicate dishes. Plates were generally incubated for 2 weeks. On the last day of incubation, 0.5 ml of p-iodonitrotetrazolium violet (0.5 mg/ml; Sigma Chemical Co., St. Louis, Mo.) was added and incubation continued for another 24 hr. The number of colonies was determined with the aid of a stereoscope (Wild) or Omnicon image analysis system (Bausch and Lomb). Colonies larger than 2400 sq μm (approximately 32 cells) were scored (37). EGF was obtained from Collaborative Research or from Bethesda Research Laboratories (Rockville, Md.); no difference in the induction of anchorage-independent growth was found using these 2 preparations.

**Assay of Plasminogen Activator.** In brief, plasminogen activator was assayed, in the presence of pure plasminogen, by the hydrolysis of labeled fibrin coated onto tissue culture wells (14, 39). Purified human fibrinogen, depleted of plasminogen, was a kind gift of Dr. John Finlayson, Bureau of Biologics, FDA, Bethesda, Md. Plasminogen was purified from fetal calf serum by affinity chromatography by the method of Quigley et al. (32). All assays used a urokinase standard, purchased from Leo Pharmaceuticals, Denmark. Determination of cell-associated and extracellular plasminogen activator was performed as described previously (13). Cells were grown in DMEM containing 10% plasminogen-depleted fetal calf serum. Cells were washed twice in serum-free medium and preincubated with EGF and/or retinoids. After 24 hr, cells were washed with serum-free medium and incubated in this medium plus EGF and/or retinoids. After 18 hr, medium was collected, centrifuged at 2000 rpm, and acidified to pH 3.5 by careful addition of 1 N HCl and then stored at −20°. Cells were removed from the dish and washed twice in phosphate-buffered saline. The pellet was resuspended in 0.25 M sucrose, 0.01 M Tris-HCl, and 0.001 M EDTA (pH 7.4). Following Dounce homogenization, the cell suspensions served as a source to determine cell-associated plasminogen activator.

**RESULTS**

**Effects of Retinoic Acid and EGF on Morphology and Cell Substratum Adhesiveness.** Two nontransformed cell lines designated NRK 536-3-1 and NRK-536-3-14 and one transformed cell line, NRK 536-3-9, were used in this study. The 2 nontransformed cell lines grow strongly contact inhibited and undergo a moderate change in morphology after treatment with retinoic acid or EGF (Fig. 1). Simultaneous treatment of these cells with retinoic acid and EGF induces a dramatic morphological change, as shown in Chart 1D. The cells appear to have extended processes and are no longer contact inhibited. The transformed NRK 536-3-9 cells are not contact inhibited and have a rounded-up or spindle-like morphology. Treatment with EGF has no effect on morphology, whereas retinoic acid (10−5 M) treatment reduces slightly the rounded-up morphology.

Treatment of the nontransformed cells with retinoic acid or EGF results in a decrease of the cell-substratum adhesiveness (Chart 1). Simultaneous treatment of these cells further reduces the cell-substratum adhesiveness. Compared to their nontransformed counterparts, the transformed cells are much less adhesive and can be removed rapidly by trypsin. Their adhesiveness is not affected significantly by either EGF or retinoic acid treatment (data not shown).

**Effect on Anchoragel-dependent Cell Growth.** Both retinoic acid and EGF stimulate cell growth of NRK 536-3-1 (Chart 2). The growth stimulation induced by retinoic acid occurs at low (10−10 M) concentrations but is optimal at 10−6 M. Both cell number and [3H]thymidine incorporation are enhanced after treatment with retinoic acid or EGF. Simultaneous addition further increases both cell number and [3H]thymidine incorporation.

**Binding of Epidermal Growth Factor.** As shown in Table 1, the 2 contact-inhibited cell lines exhibit a relatively much higher level of 125I-EGF binding than do the transformed NRK-536-3-9 cells. Treatment of NRK 536-3-1 and NRK 536-3-14 with either retinoic acid or retinol enhances the binding of 125I-EGF several-fold, whereas treatment of the transformed cells with retinoids does not affect this binding. The stimulation of EGF binding reaches a maximum level at a concentration of 10−6 M retinoic acid. The biologically inactive pyrimidyl analogue of retinoic acid exhibits only a small effect on the binding of EGF.

**Anchorage-independent Growth.** The NRK 536-3-9 cells have a strong ability to form colonies in semisolid medium (Table 2). This ability is not markedly influenced by either the presence of EGF or retinoic acid. The 2 nontransformed NRK cell lines remain mostly as single cells when suspended in soft agar. However, anchorage-independent growth of these cells is induced by the addition of EGF (Table 2). Simultaneous treatment
In independent experiments. The variation was not larger than 5%.

Binding studies were carried out in duplicate dishes. Results are from 2 independent experiments. The variation was not larger than 5%.

In the binding assay, 251-labeled mouse EGF, 0.85 ng/ml (9.5 x 10^6 dpm/ng), was used. Binding studies were carried out in duplicate dishes. Results are from 2 independent experiments. The variation was not larger than 5%.

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of retinoic acid, can stimulate colony formation; however, the biologically inactive pyrimidyl analogue of retinoic acid is much less active (not shown).

with EGF and retinoic acid enhances the colony formation dramatically, whereas retinoic acid alone has no effect.

Chart 3 shows anchorage-independent growth of NRK-536-3-1 cells as a function of the EGF concentration. Soft agar assays were carried out as described in "Materials and Methods." The number of cells per 60-mm dish was 10^4. Retinoic acid was used at a concentration of 10^-6 M. O, EGF only; O, EGF plus retinoic acid. Experiment was carried out in duplicate. Variation was smaller than 10%.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>Binding of 125I-EGF (10^6 dpm/10^4 cells)</th>
<th>Relative binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRK 536-3-1</td>
<td>Control</td>
<td>11.7</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>+ retinoic acid</td>
<td>36.8</td>
<td>3.14</td>
</tr>
<tr>
<td></td>
<td>+ retinol</td>
<td>23.9</td>
<td>2.04</td>
</tr>
<tr>
<td></td>
<td>+ pyrimidyl analogue of retinoic acid</td>
<td>14.6</td>
<td>1.25</td>
</tr>
<tr>
<td>NRK 536-3-14</td>
<td>Control</td>
<td>9.6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>+ retinoic acid</td>
<td>21.4</td>
<td>2.24</td>
</tr>
<tr>
<td></td>
<td>+ retinol</td>
<td>14.9</td>
<td>1.56</td>
</tr>
<tr>
<td>NRK 536-3-9</td>
<td>Control</td>
<td>0.03</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>+ retinoic acid</td>
<td>0.03</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>+ retinol</td>
<td>0.03</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>No. of colonies/60-mm dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRK-536-3-1</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>+ EGF</td>
<td>336</td>
</tr>
<tr>
<td></td>
<td>+ retinoic acid</td>
<td>4103</td>
</tr>
<tr>
<td></td>
<td>+ EGF + retinoic acid</td>
<td>4103</td>
</tr>
<tr>
<td>NRK-536-3-14</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>+ EGF</td>
<td>671</td>
</tr>
<tr>
<td></td>
<td>+ retinoic acid</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+ EGF + retinoic acid</td>
<td>5788</td>
</tr>
<tr>
<td>NRK-536-3-9</td>
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<tr>
<td></td>
<td>+ EGF</td>
<td>8602</td>
</tr>
<tr>
<td></td>
<td>+ retinoic acid</td>
<td>8991</td>
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</table>

Chart 4. Effect of EGF and retinoic acid on anchorage-independent growth of NRK-536-3-1 cells induced by EGF and retinoic acid. Colony formation was measured as a function of cell density. Conclusions of EGF and retinoic acid were 0.3 ng/ml and 10^-6 M, respectively. O, EGF only; O, EGF plus retinoic acid. Experiment was carried out in duplicate. The difference between number of colonies was no larger than 10%.

<table>
<thead>
<tr>
<th>No. of colonies formed in the presence of</th>
<th>Fold enhancement (RA + EGF/EGF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells tested</td>
<td>No addition</td>
</tr>
<tr>
<td>NRK-536-3-1</td>
<td>0</td>
</tr>
<tr>
<td>E2</td>
<td>0</td>
</tr>
<tr>
<td>E3</td>
<td>0</td>
</tr>
<tr>
<td>E4</td>
<td>0</td>
</tr>
<tr>
<td>E1</td>
<td>0</td>
</tr>
<tr>
<td>E2</td>
<td>0</td>
</tr>
<tr>
<td>ER3</td>
<td>0</td>
</tr>
<tr>
<td>ER4</td>
<td>0</td>
</tr>
<tr>
<td>ER6</td>
<td>0</td>
</tr>
</tbody>
</table>

*RA, retinoic acid.

Reversibility of Anchorage-independent Growth. In order to determine whether the anchorage-independent growth property is reversible, several colonies formed in soft agar were isolated, subcultured without EGF or retinoic acid, and retested for their...
536-3-9 cells are not contact inhibited, exhibit low EGF binding and low cell substratum adhesiveness, produce large amounts of plasminogen activator, and grow progressively in soft agar. These properties are not markedly affected by the addition of either retinoids or EGF. In contrast, the 2 nontransformed cell lines are strongly contact inhibited, exhibit relatively high EGF binding, produce very low levels of plasminogen activator, and do not form colonies in soft agar. Treatment of these cells with retinoic acid stimulates anchorage-dependent growth and causes a reduction in cell substratum adhesiveness. Moreover, this compound enhances the binding of EGF 2- to 3-fold but does not affect anchorage-independent growth or the production of plasminogen activator. Treatment with EGF increases anchorage-dependent growth and reduces cell substratum adhesiveness. Addition of EGF induces anchorage-independent growth, and this property is enhanced dramatically by the simultaneous presence of certain retinoids. EGF also increases the production of plasminogen activator, but this response is not affected by retinoids. A stimulation of plasminogen activator by EGF has been reported earlier by Lee and Weinstein (25). At least a part of the enhancement of anchorage-independent growth by retinoids may be due to the increased EGF binding. Retinoic acid treatment increases EGF binding almost 3-fold; however, the EGF-induced anchorage-independent growth is enhanced more than 10-fold by this compound. This indicates that the effect on growth in soft agar is greater than can be expected only on the basis of the increased EGF binding, suggesting that additional factors may play a role. The increase in sarcoma growth factor-induced growth in agar by retinoic acid, reported previously (21), may also be partially related to the enhanced EGF binding.

It is important to emphasize that the action of retinoids on anchorage-independent growth is clearly distinct from that of EGF, in that EGF induces anchorage-independent growth, whereas retinoic acid alone has no effect but potentiates EGF-mediated anchorage-independent growth. The potentiating ability of retinoids may be brought about via an enhancement in the inductive process (e.g., via increased EGF binding) or via an alteration in the expression of growth in soft agar. The stimulation of cell growth and the decreased cell substratum adhesiveness may reflect alterations which interfere with the expression of the transformed phenotype independent of the inducer.

It has been shown that EGF can magnify anchorage-independent growth of virus-transformed cells (12), epithelial cells (9), and human tumor cells (16). Recently, it has been reported that in combination with certain transforming growth factors, which by themselves induce anchorage-independent growth very poorly, addition of EGF can enhance the ability to form colonies in soft agar (33). Low levels of such transforming growth factors produced by the nontransformed rat kidney cells (38) or present in serum (3) may, in combination with EGF, induce anchorage-independent growth. If that is the case, this opens the possibility that retinoids may interfere with the mechanism of action of these transforming growth factors.

It seems apparent that changes in the components of the cellular matrix are involved in the anchorage-independent growth (17). These components can be affected via alterations in their synthesis or their glycosylation, which may influence the secretion, sensitivity toward proteolytic degradation, or interaction with other matrix components. Alternatively, these components

**DISCUSSION**

This paper describes the interaction of EGF and retinoids on various parameters of 3 NRK cell lines. The transformed NRK cells tested were obtained from colonies induced either by simultaneous treatment of retinoic acid and EGF or by EGF treatment alone. As shown in Table 3, like the original NRK 536-3-1 cells, none of the cells formed colonies in soft agar spontaneously or in the presence of retinoic acid. Anchorage-independent growth can be induced by EGF treatment and potentiated by the simultaneous presence of retinoic acid. The responses to EGF and retinoic acid show some heterogeneity. Anchorage-independent growth is poorly induced by EGF in ER1 and E3, whereas EGF is very effective in E2. Retinoic acid is very effective in ER1 and E3 but enhances colony formation in E2 only 2-fold. However, this effect of retinoic acid in E2 becomes more apparent when only the colonies larger than 6900 sq. µm are counted: in EGF-treated cells, only 171 colonies are counted, whereas treatment with EGF and retinoic acid gives rise to 3128 colonies.

**Production of Plasminogen Activator.** The production of plasminogen activator in NRK cells has been implicated in the ability of certain cells to grow in semisolid medium. Therefore, we examined the effects of EGF and retinoic acid on the production of this protease. As shown in Chart 5, addition of EGF enhances the extracellular plasminogen activator activity by almost 3-fold. Addition of retinoic acid does not alter the secretion of plasminogen activator in either the presence or the absence of EGF. A more extensive study using 10^-6 to 10^-8 M retinoic acid and incubation periods of 6 hr to 4 days did not reveal an effect on plasminogen activator secretion in these cells. A similar result as for the extracellular plasminogen activator activity was obtained for the cell-associated activity. Although EGF increases plasminogen activator activity in nontransformed NRK cells, the levels of this enzyme remain relatively low compared to that of the transformed NRK 536-3-9 cells. Plasminogen activator activity in transformed cells is about 20 times higher than that in nontransformed cells treated with EGF.
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can be affected via changes in the synthesis or activation of certain proteolytic enzymes. One such enzyme that has been implicated in the mechanism of anchorage-independent growth is plasminogen activator (25, 27, 29, 31). Although it has been demonstrated that retinoids can enhance the production of this enzyme in certain cells (34, 40), this production is not affected in the nontransformed rat kidney cells that we tested. Retinoids may increase anchorage-independent growth by enhancing the production of other proteases or inducing changes in certain cell surface components.

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Fig. 1. Effect of retinoic acid and EGF on the morphology of NRK 536-3-1 cells. Cells were grown in DMEM containing 1% fetal calf serum and treated for 3 days with ethanol in the control (A), $10^{-6}$ M retinoic acid (B), EGF, 5 ng/ml (C), and $10^{-6}$ M retinoic acid plus EGF, 5 ng/ml (D).
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