Multistep Change in Epidermal Growth Factor Receptors during Spontaneous Neoplastic Progression in Chinese Hamster Embryo Fibroblasts

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ABSTRACT

Whole Chinese hamster embryo lineages have been shown to undergo multistep spontaneous neoplastic progression during serial passage in culture. We have studied the binding, internalization, and degradation of 125I-labeled epidermal growth factor at four different stages of transformation. The whole Chinese hamster embryo cells lost cell surface epidermal growth factor receptors gradually during the course of neoplastic progression until only 10% of the receptor number present in the early-passage cells (precrisis) were retained in the late-passage cells (tumorigenic). No differences in internalization rates, chloroquine sensitivity, or ability to degrade hormone between the various passage levels were seen. No evidence for the presence in conditioned medium of transforming growth factors which might mask or down-regulate epidermal growth factor receptor was obtained. These results suggest that a reduction in cell surface epidermal growth factor receptor might be an early event during spontaneous transformation in whole Chinese hamster embryo cells.

INTRODUCTION

The awareness that neoplasias do not arise in a single step with a completely expressed transformed-tumorigenic phenotype but rather develop in a progressive stepwise fashion has existed most of this century (17, 18). Recent work with in vitro models of spontaneous and chemically induced transformation has shown that the tumorigenic stage is preceded by morphological alterations, enhanced fibrinolytic activity, reduced serum requirement, and anchorage-independent growth (2-5, 27, 40).

The reduction in serum requirements for growth of transformed cells has been attributed to a decreased requirement for serum-derived growth factors (6, 11, 24, 39, 41, 42, 44). Virally and chemically transformed fibroblasts have a reduced EGF requirement for growth (6, 11, 47). However, there is no simple relationship between the expression of EGFRs and the degree of transformation. Transformed cell lines have displayed either a decrease (7, 30, 32, 44), no change (7), or an increase (15, 25, 43) in EGFR expression as measured by their ability to bind radiolabeled ligand. In some cases, a decreased level of surface EGFR has been correlated with the autocrine production of transforming growth factors which can have a component of EGF-like activity (1, 13, 14, 30). Wharton et al. (48) have correlated a decrease in EGF binding with decreased EGF requirement for growth in rodent fibroblast lines but not in human fibroblasts.4 These results point out the need for caution when trying to establish generalized cause and effect relationships between transformation, growth factor requirements, and growth factor receptor expression.

Recently, we have established a series of cell lineages from WCHE which have been shown to undergo spontaneous neoplastic progression during serial propagation in vitro (27). By using a battery of transformation assays, we have defined 4 stages in the progression toward a tumorigenic phenotype (see "Results") and have correlated the altered phenotypes with karyotypic abnormalities (12). Since loss of EGF binding in certain virally transformed fibroblast cells has been one of the earliest markers noted (44), the availability of cells at various stages of transformation made investigation of the EGF binding and processing capabilities of the WCHE cell lineage desirable. In this report, we present data showing a gradual decrease in EGF expression during the course of neoplastic progression. A preliminary report of this study has appeared (46).

MATERIALS AND METHODS

Cell Culture. The WCHE cell lineage used in this study (WCHE/5) was one of several established by trypsinization of whole embryos and subsequent serial passage (see Ref. 27 for details). Cells were frozen in liquid nitrogen at various passage levels and thawed when needed. Cells from any one freeze-down vial were used for 3 to 4 passages. Cultures were maintained in α-minimum essential medium (Grand Island Biological Co.) containing 10% fetal calf serum (Reheis Chemical Co.). Cells were grown in 35-mm tissue culture dishes (Corning) for binding experiments. Cells used for experiments were plated at an initial density of 50,000 cells/dish and used 3 to 5 days later. All cells used were negative for Mycoplasma contamination.

Binding of 125I-EGF. EGF purified from mouse salivary glands by the method of Savage and Cohen (37) was a kind gift of Dr. E. J. O'Keefe. 125I-EGF was prepared by the chloramine-T method (23); specific activity was generally 1 to 2 × 10^6 cpm/μg.

All binding experiments were performed on intact cells in 35-mm tissue culture dishes. Cultures were cooled on ice, rinsed once with 1 ml binding buffer (HBSA), and then incubated with HBSA plus 125I-EGF for 2 to 4 × 10^5 cpm/ml; 1 to 2 ng) at the appropriate temperature. For the chloroquine experiments, 100 μM chloroquine was included in the preincubation as well as the 125I-EGF-containing incubation medium. At the end of the incubation, unbound radioactivity was removed by rinsing 5 × 1 ml ice-cold HBSA. The cell-associated radioactivity was then removed with 1 ml 0.2 N NaOH and counted in a Packard Auto-Gamma scintillation spectrometer. Nonspecific binding was determined in the presence of 500-fold excess unlabeled EGF. This usually amounted to 5 to 10% of the total counts at 37°C and 15 to 25% at 4°C. For the equilibrium binding experiments, specific binding was corrected for cell number.

4 W. Wharton and W. J. Pledger, unpublished observations.

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Internalization and Degradation of Cell-bound 125I-EGF. Cultures were prelabeled with 125I-EGF at 4°C for 3 to 4 h, rinsed free of unbound label, and then incubated in 0.6 ml HBSSA at 37°C. At appropriate times, cultures were cooled on ice, and the medium was removed and combined with a 0.4-ml rinse of ice-cold HBSSA. Cell surface 125I-EGF was removed by incubation in 1 ml of 0.2 M acetic acid-0.25 M NaCl (pH 3.0) for 5 min on ice (21). The remaining radioactivity (cell associated) was removed with 1 ml 0.2 M NaOH.

To determine the amount of 125I-EGF degraded by the cells, cultures were prelabeled as described above and incubated with or without 100 µM chloroquine in HBSSA at 37°C. At the appropriate time, the medium was removed, and a 0.5-ml sample was chromatographed over a P-4 column (0.8 x 20 cm; Bio-Rad) equilibrated with 100 mM Na2HPO4, pH 7.4. The same buffer was used to elute the sample. Fifteen-ml fractions were collected and counted.

Cell numbers were determined by counting trypsinized cellular monolayers with a Coulter Counter (Coulter Electronics, Inc.).

RESULTS

Binding of 125I-EGF at 37°C and 4°C. Four passage levels of WCHE/5 cells, representing the sequential steps of neoplastic progression as defined previously (27), were tested for their ability to bind and accumulate 125I-EGF. The different stages are defined briefly as follows: Stage I, P9 to 11, diploid, nontransformed, nontransformed conditionally tumorigenic (precrisis); Stage II, P15 to 19, immortalized (postcrisis), aneuploid, nontransformed, conditionally tumorigenic; Stage III, P30 to 33, partially transformed, nontumorigenic; Stage IV, P50 and beyond, highly transformed and tumorigenic. The results show that the cells of different passage levels were able to accumulate strikingly different amounts of 125I-EGF when incubated at 37°C (Chart 1). In this particular experiment, reduced EGF accumulation was apparent with Stage II cells (P19) that were yet untransformed by conventional assays.

In order to determine whether the differences in hormone accumulation at 37°C between the various passages were due to alterations in the number of cell surface EGFRs, cells were exposed to increasing concentrations of 125I-EGF at 4°C (to eliminate postbinding processing steps) until equilibrium binding was achieved (6 to 7 h). The data were analyzed by the method of Scatchard (38). The results are shown in Chart 2, and the binding parameters (Kd and Bmax) derived from the Scatchard analysis are presented in Table 1. There was essentially no change in the affinity of the receptor for its ligand (1 to 2 nM) between the various passages, whereas the number of surface receptors per cell decreased with increasing passage number. Thus, the late-passage tumorigenic cells (P72) have approximately 10% of the number of surface receptors as the precrisis (P9) WCHE cells. Such a decrease in EGFR number has been described in chemically (20, 22, 36) and virally (13, 44) transformed cells, as well as in a rat cell line spontaneously transformed in vitro. To our knowledge, this is a first report of a decrease in EGFR number in cells established in primary cell culture and transformed spontaneously in vitro.

Although the decrease in 125I-EGF binding between the early-precrisis (Stage I) and very-late-passage (Stage IV) cells was a consistent finding during the course of these investigations, variability in relative receptor levels was seen in the intermediate passage levels, particularly in the immediately postcrisis (Stage II) cells. Table 2 shows the amount of 125I-EGF bound at 4°C to cells of different passage levels which were derived from freeze-down vials thawed separately from those used to obtain the data in Chart 2. In this case the P19 cells bound more hormone than

![chart](chart1.png)

Chart 1. Accumulation of 125I-EGF at 37°C. Cultures were incubated for 180 min in labeled EGF (3 x 10^5 cpm/dish) in HBSSA for the time indicated and then processed as described in "Materials and Methods." Data expressed as cpm/cell.

![chart](chart2.png)

Chart 2. Scatchard analysis of equilibrium binding data. Cells were incubated 6 to 7 h at 4°C with increasing amounts of 125I-EGF (range, 0.1 to 20 nM). At the end of the incubation period, 100-µl aliquots of the incubation medium was taken for determination of free ligand concentration. Cultures were then rinsed and processed as described in "Materials and Methods." Binding data were corrected for cell numbers. The B/F (bound/free) units are (ml/cell) x 10^-6.

<table>
<thead>
<tr>
<th>Passage</th>
<th>Kd (nM)</th>
<th>Bmax (no. of receptors/cell)</th>
<th>r</th>
<th>No. of cells/cm² x 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>P9</td>
<td>2.08</td>
<td>50,000 (100)</td>
<td>0.951</td>
<td>0.586</td>
</tr>
<tr>
<td>P18</td>
<td>1.76</td>
<td>29,500 (59)</td>
<td>0.913</td>
<td>0.423</td>
</tr>
<tr>
<td>P33</td>
<td>1.65</td>
<td>16,800 (34)</td>
<td>0.903</td>
<td>2.340</td>
</tr>
<tr>
<td>P72</td>
<td>1.26</td>
<td>3,850 (8)</td>
<td>0.903</td>
<td>5.585</td>
</tr>
</tbody>
</table>

* Data derived by linear regression analysis of data in Chart 2.
* Numbers in parentheses, percentage.

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Table 2
Effect of cell density on 125I-EGF binding at 4°C

<table>
<thead>
<tr>
<th>Passage</th>
<th>No. of cells/dish x 10^6</th>
<th>cpm/cell x 10^-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>P9</td>
<td>0.465</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>0.664</td>
<td>4.1</td>
</tr>
<tr>
<td>P19</td>
<td>0.177</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>0.214</td>
<td>7.6</td>
</tr>
<tr>
<td>P31</td>
<td>0.697</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>2.020</td>
<td>1.7</td>
</tr>
<tr>
<td>P71</td>
<td>2.696</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>5.888</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Attempts were made to determine whether the presence of transforming growth factors produced by cells undergoing neoplastic progression might account for the decrease in EGFRs. Such factors have been shown to be present in the culture medium of virally (13, 14, 45) and chemically (30) transformed cells, as well as in nonneoplastic tissue (33, 35). Early-passage (P9) cells were incubated for up to 24 h with medium conditioned by confluent P73 cells for 48 to 72 h. Binding of 125I-EGF at 4°C or 37°C to P9 cells thus treated was unchanged from untreated control cultures. No attempt was made to concentrate or purify any putative conditioned medium factors.

Contribution of Cell Density to Reduced 125I-EGF Binding. The density of the WCHE cells at confluence and the ability to grow after confluence has been reached (loss of contact inhibition) increased with postcrisis passage level (Ref. 27; see Tables 1 and 2). Previous reports have documented the decrease in 125I-EGF binding per cell as a function of increased cell density (6, 7). We were unable to measure hormone binding in the different passages at the same cell density due to the fact that large areas of the culture dish were exposed in late-passage cultures (because of a small decrease in cell size and cell overlap) at densities comparable to confluent early-passage cultures. Under these circumstances, the increased nonspecific binding to the exposed culture dish masked the already small binding signal of the late-passage cultures. We therefore measured the binding of 125I-EGF to the different passages at each of 3 densities. The data obtained from such an experiment (Chart 5) show that cell density can account for some but not all of the decreased 125I-EGF binding. Note that the lines are neither coincident nor parallel and thus describe different functions of cell density on the expression of 125I-EGF binding. The effect of cell density appears most profound in the early-passage cultures and decreases with increased passage number.

DISCUSSION

Our WCHE cell lineages have been shown to pass through a number of stages during spontaneous neoplastic progression, leading ultimately to a transformed and tumorigenic phenotype (27). In this report, we have documented the gradual loss of EGF binding to WCHE/5 cells with increasing passage level. This loss is due to a decreased expression of EGFR at the cell surface. Whether there is a compensatory alteration of EGFR within internal pools has not been determined, but the 37°C accumulation data suggest that, if such internal receptors exist, they remain functionally quiescent. The different passage levels appear to have unaltered processing capabilities as judged by their internalization rates, sensitivity to chloroquine, and ability to degrade hormone.

It seemed possible that the loss of EGFR could have been due to a transforming growth factor-like activity secreted by the cells in progressively larger amounts in later-passage cells. Loss of EGF-binding activity due to such factors has been reported (13, 14, 30, 45). However, incubation of early-passage cells with conditioned medium from late-passage cultures did not alter their binding of 125I-EGF. This result does not rule out a true autocrine stimulation whereby a cell could secrete a factor which stimulates itself but not neighboring cells. Neither can we eliminate the possibility that some factor is produced, binds to intracellular EGFR, and either masks their presence at the surface and

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Chart 3. Internalization of prebound 125I-EGF. Cultures were prelabeled with 2.5 x 10^5 cpm/dish 125I-EGF for 3 h at 4°C, rinsed, and then incubated at 37°C. At the appropriate time, cultures were cooled on ice, the medium was saved, and the surface-bound label was removed with an acid wash (see "Materials and Methods"). Cell-associated radioactivity was removed with 0.2 N NaOH. Results are expressed as a percentage of the total radioactivity (medium + acid wash + cell-associated) present in each fraction. The average total radioactivity per dish was as follows: P9, 6,484 cpm; P20, 2,922 cpm; P31, 5,984 cpm; P73, 4,772 cpm. The standard deviations were less than 10% of the total.

Table 3

Chloroquine-enhanced accumulation of 125I-EGF at 37°C

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>P11</th>
<th>P19</th>
<th>P33</th>
<th>P73</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>1.27</td>
<td>0.94</td>
<td>0.96</td>
<td>1.05</td>
</tr>
<tr>
<td>45</td>
<td>0.97</td>
<td>0.91</td>
<td>0.90</td>
<td>0.87</td>
</tr>
<tr>
<td>60</td>
<td>0.94</td>
<td>0.94</td>
<td>0.81</td>
<td>0.79</td>
</tr>
<tr>
<td>120</td>
<td>0.57</td>
<td>0.72</td>
<td>0.58</td>
<td>0.58</td>
</tr>
<tr>
<td>140</td>
<td>0.35</td>
<td>0.41</td>
<td>0.36</td>
<td>0.39</td>
</tr>
</tbody>
</table>

induces their internalization or prevents their expression at the surface entirely. The lack of involvement of a soluble factor in the reduction of EGFR expression in virally or chemically transfected cells has been reported (20, 22). Changes in acetylcholine and insulin receptor levels during denervation-induced hypersensitivity and differentiation, respectively, have been ascribed to increased receptor biosynthesis (16, 34), while ligand-induced down-regulation of insulin and EGF receptors involved increased rates of inactivation/decay (26, 28). We do not yet know at what step (gene expression, receptor turnover) the altered level of EGFR expression in late-passage cells is regulated. Although the loss of EGFR was a consistent finding throughout the course of these investigations, variability in EGF binding was noted between different frozen stocks of the various passages, as if the process of freeze-down into liquid nitrogen and subsequent thawing might be the cause of a selection process in some cases. This was particularly true of the immediately postcrisis cells (P18 or Stage II), which had EGF binding characteristics
that fluctuated between the precrisis cells (P9) or greater, to about the same as much later passages (P30 or Stage III). Coincident with this fluctuation were morphological changes (data not shown) which correlated with receptor levels (i.e., increased EGFR-normal morphology; decreased EGFR-transformed morphology). Thus, within this variability was the consistent finding that low EGFR levels correlated with the transformed phenotype.

This variability may in fact be a reflection of the type of selection process necessary for neoplastic progression, whereby the cell subtype with the fastest doubling time and least stringent growth requirements ultimately outgrows all other cells in the environment. Such a scenario presupposes some degree of cellular heterogeneity. Particularly relevant in this regard are reports showing heterogeneity of thrombin (8) and transferrin (31) receptor levels within a "homogeneous" population of cells. EGFR levels in the WCHE cell lines might prove to be an early marker for cells undergoing spontaneous transformation, as has been suggested for virally transformed cells (44). It should be possible to test this hypothesis by selecting subpopulations of cells with varying EGFR levels, using fluorescence-activated cell-sorting techniques. Transformation indicators and the tumorigenic potential of the differing populations of cells could then be ascertained by standard assay methods.

In conclusion, we have found a correlation between decreased surface EGFR and increased passage levels in a WCHE cell line undergoing spontaneous neoplastic progression. Although no cause and effect relationship between these 2 phenomena has been established, EGFR levels might prove to be a useful early marker for the neoplastic process.

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