Human Esophageal Carcinoma Cells Have Fewer, but Higher Affinity Epidermal Growth Factor Receptors*

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Squamous cell carcinomas have recently been shown to contain increased numbers of epidermal growth factor (EGF) receptors. Since EGF has an important role in epithelial growth and differentiation, it is possible that modulation of its receptor may have an important role in neoplasia. In an attempt to further explore the relationship of EGF receptor expression to malignant transformation, we examined 14 squamous cell carcinoma cell lines of the esophagus for the number and affinity of EGF receptors. Seven cell lines were newly isolated by this laboratory and recently characterized. The seven additional cell lines were obtained from Japan (4 cell lines) and South Africa (3 cell lines). Surprisingly, we found that esophageal carcinomas contained lowered quantities of surface EGF receptors (2-100-fold) and that the affinity of the EGF receptor was increased (6-100-fold) when compared to normal esophageal epithelial cells. Moreover, the biological response of esophageal carcinoma cells to EGF differed markedly from that of other squamous cell tumor cells exhibiting elevated numbers of receptors, such as A431 and SCC-15. Human esophageal carcinoma cells were maximally stimulated by the addition of 5 ng/ml of EGF, similar to normal esophageal keratinocytes, but in contrast to normal cells were not inhibited by the higher concentrations tested (up to 40 ng/ml). On the other hand, addition of any EGF to the medium (beyond that normally present in serum) was found to dramatically inhibit the growth of A431 and SCC-15 cells. Our findings indicate that squamous cell neoplasia is not dependent upon increased numbers of cell surface EGF receptors, that EGF receptor number may have a determinant role in EGF cell toxicity, and that the stimulatory response of cells to EGF may reflect a complex function of EGF receptor number, affinity, and occupancy.

Epidermal growth factor (EGF) binds to specific saturable membrane receptors on the surface of a wide variety of cell types in cell culture and regulates numerous cellular functions (1, 2). Most normal murine and human cells have $10^3$-10$^4$ receptors/cell (3, 4) although normal squamous epithelial cells such as epidermal cells generally exhibit higher levels (2.5 x 10$^4$ receptors/cell) (5). EGF and its receptor are important regulators of epidermal growth and differentiation. EGF is mitogenic for normal keratinocytes in cell culture while inhibiting differentiation (6). Squamous cell carcinomas in cell culture have been reported to express 5-50-fold more EGF receptors per cell than do normal keratinocytes (5) and similar increases have been observed on squamous cell carcinoma biopsy materials derived from lung, head and neck, skin, and cervix (5, 7, 8), suggesting elevated numbers of EGF receptors may represent an important step in neoplastic progression. Perhaps a reflection of the large numbers of EGF receptors, squamous cell carcinoma cell lines (such as A431) have been found to be maximally stimulated by very low concentrations of EGF which would be inhibitory to the growth of normal keratinocytes (5).

Because EGF is an important regulator of normal squamous epithelial growth and differentiation and because EGF receptor number has been shown to be elevated in all squamous cell carcinoma cells examined thus far, thus suggesting its possible role in malignant growth, we sought to investigate whether EGF might have differential effects on normal and transformed esophageal epithelial cells and whether the EGF receptor was altered in the transformed cells. Using cell lines isolated by our laboratory (9), as well as those obtained from others (10-13), we found two important and interesting differences in the EGF receptors of esophageal carcinoma cells which don't correlate with previous findings for squamous cell carcinomas from other anatomic sites; esophageal cells have fewer receptors with increased ligand affinity. Moreover, these esophageal tumor cells with fewer receptors are much more tolerant of increased levels of EGF compared to squamous tumors with high levels of EGF receptor.

MATERIALS AND METHODS

Human esophageal epithelial cells and esophageal carcinoma cell lines (HCS series) were isolated and grown as described previously (5, 14). Human esophageal carcinoma cell lines designated HCU and TE were generously provided by Dr. Kathy Robinson, Department of Physiology, University of Natal, Congella, South Africa, and by Drs. Morio Kasai, Tetsuro Nishihira, and Takashi Akaike of the Second Department of Surgery, Tohoku University School of Medicine, Sendai, Japan, respectively, and the growth characteristics have been described previously (10-13). The HCU cell lines were grown in Medium 199 plus 8% fetal bovine serum and TE cell lines in RPMI 1640 containing 10% fetal bovine serum. Cell lines A431 and SCC-15 were obtained from the American Type Culture Collection, Rockville, MD, and maintained in RPMI 1640 with 10% fetal bovine serum or Medium 199 with 20% fetal bovine serum, respectively. All other cell lines (HCE series) were established in the laboratory of one of the authors (S. P. B.-S.).

Routine binding assays were performed by a modification of the method of Richert et al. (15). Briefly, nearly confluent (80-90%) cultures of normal and transformed human esophageal epithelial cells, grown in 35-mm tissue culture dishes, were washed twice and then incubated in 2 ml of serum-free Medium 199 containing 20 mM Hepes, pH 7.5. Following 1 h at 37°C, duplicate dishes were then incubated at 4°C for an additional 45 min, the medium was aspirated, and 2 ml of binding medium (Medium 199 containing 20 mM Hepes, pH 7.5, 0.1% bovine serum albumin (fraction V, Miles Laboratories, Inc., Naperville, IL), and the appropriate concentration of $[^{125}]$-EGF (spe-
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cific activity 178 Ci/µg, New England Nuclear) was added. Non-specific binding was measured in the presence of a 1000-fold excess unlabeled EGF (receptor grade, Bethesda Research Laboratories) and was <5% of the total radioactivity bound. After the appropriate time of incubation at 4°C, the cells were washed four times with 2 ml of ice-cold binding medium (without EGF), solubilized with 1 ml of 1 M NaOH at 37°C, and transferred to a 12 x 75-mm plastic tube for counting in a γ-counter. Kinetic experiments were performed at a concentration of 1 nM (6 ng/ml) ¹²⁵I-EGF. For dose-response experiments, increasing concentrations of ¹²⁵I-EGF in the range of 0.01 to 500 nM were used, the upper limits depending on the cell or cell line. In the case of very high concentrations of ¹²⁵I-EGF (100 nM or greater) non-specific binding was performed in the presence of 100-fold instead of 1000-fold excess unlabeled EGF. Total cell count on triplicate dishes, set up in parallel with the experimental dishes, was determined using a hemocytometer chamber.

RESULTS

EGF Receptor Level and Affinity in Normal and Transformed Esophageal Epithelial Cells—To determine the EGF receptor level and affinity of the receptors in normal and transformed esophageal keratinocytes, cells were incubated with ¹²⁵I-EGF at 4°C. Fig. 1 shows the kinetics of EGF binding to normal human esophageal keratinocytes and a representative esophageal carcinoma cell line at 4°C. The measurements have been corrected for non-specific binding as described under “Materials and Methods.” Whereas it took 12 h to reach saturation in the case of normal esophageal cells, 6-fold less time or only 1-2 h was required to reach steady state in the case of the esophageal carcinoma cells. The concentration dependence of ¹²⁵I-EGF binding to normal esophageal cells and two representative cell lines is shown in Fig. 2. Binding of ¹²⁵I-EGF to the cells was performed at 4°C for 16 h in the case of the normal cells and for 4 h in the case of the transformed cells to ensure steady state had been reached. Normal esophageal cells bound much more EGF than did the carcinoma cells. Maximal binding occurred at

approximately 25 nM in the case of the normal cells whereas in the transformed cells it was around 1 to 6 nM EGF. Scatchard analyses of these data, shown in Fig. 3, demonstrated a single class of receptors to be present in both normal and transformed esophageal cells. The experimental values were fit to a single line using linear regression and all lines had correlation coefficients greater than 0.92. A summary of the estimated affinity and number of EGF receptors on normal and transformed esophageal cells is shown in Table I. We found two important and interesting differences in the EGF receptors of esophageal carcinoma cells that don’t correlate with what others have found for other squamous cell carcinomas (in vivo and cell culture) thus far, namely they have fewer receptors with an increased ligand affinity. In contrast to normal esophageal keratinocytes with approximately 400,000 EGF receptors/cell, esophageal carcinoma cells had 2- to 100-fold fewer receptors and exhibited a 6- to
coefficients greater than fit to a single line using linear regression and all lines had correlation both from that of normal esophageal keratinocytes as well as of other squamous cell carcinoma cell lines, such as A431.

Similar concentrations of EGF (namely 5 ng/ml) were not inhibited by concentrations of EGF mitogenic for normal keratinocytes. Our results suggest an interesting correlation between EGF receptor number and the biologic behavior of the cells, namely that, whereas cells with increased numbers of receptors are inhibited by concentrations of EGF mitogenic for normal cells (5), cells with fewer receptors are more tolerant of increased levels of EGF. Moreover, our studies show that the presence of high levels of EGF receptors is not always a hallmark of squamous cell malignancies and that squamous tumors can and do arise without an elevation in EGF receptor levels or markedly altered biologic responses to EGF. In this regard, most of the esophageal carcinoma lines examined in this study, which were derived from human tumors, were tumorigenic when injected subcutaneously into athymic nude mice (10), even HCE-3 which possessed 100-fold fewer receptors than normal esophageal epithelial cells and was maximally stimulated to grow by concentrations of EGF similar to that found for normal keratinocytes.

An apparent loss of EGF-binding sites on the surfaces of transformed cells (16–20) has been noted previously and one study (20) demonstrated a correlation between decreased EGF receptor levels and the stage of neoplastic progression. In some, but not all cases, the decreased level of surface EGF receptor correlated with the autocrine production of transforming growth factors (TGF) (17, 21–24). Two findings indicate that the decrease in EGF receptors on esophageal carcinoma cells is not due to autocrine TGF production. First, cells producing TGF usually do not bind EGF (18, 22) and are unresponsive to EGF in the medium (18, 25, 26). The fact that the esophageal carcinoma cells can bind BGF and are maximally stimulated to grow by concentrations of EGF required to optimally grow normal cells makes this possibility seem unlikely. Second, incubation of normal cells or A431 cells with conditioned medium from the carcinoma cells did not reduce their binding of 125I-EGF (results not shown).

In the present study, we found no simple relationship between receptor number (capacity), affinity, occupancy, and the growth-stimulatory response to EGF. Maximal growth stimulation of normal and malignant cells occurred at sub-saturating concentrations of EGF (when 10% of the cell surface receptors were occupied in the case of normal cells and when 60 to 80% of the cell surface receptors were occupied in the case of the esophageal carcinoma cells). Obviously, this means that different numbers of receptors are being occupied on normal and transformed cells in order to achieve a biologic response and that it is not easy to reconcile cell stimulation as the direct biochemical consequence of cell receptor activity. Our laboratory is currently studying potential modifications of the EGF receptor in esophageal carcinoma cells as a means of identifying the etiology of its increased affinity for EGF to normal squamous epithelial growth and differentiation (6) and because the presence of elevated levels of EGF receptors in squamous cell carcinomas derived from a variety of sources (skin, head and neck, lung, and cervix) suggested its possible role in malignant growth of these cells (5, 7, 8), we examined 14 human esophageal carcinoma cell lines for alterations in the biologic response to EGF or the EGF receptor level compared to normal esophageal keratinocytes. The data presented in this report show that transformed esophageal cells have an altered growth response to EGF and have fewer receptors with an increased affinity for binding for EGF. These findings contrast with those of other laboratories (5, 7, 8) demonstrating first, increased levels of EGF receptor in all squamous cell carcinomas (from skin, head and neck, lung, and cervix) examined thus far, both in cell lines and biopsy specimens, and secondly, growth inhibition of squamous epithelial cell lines by concentrations of EGF mitogenic for normal keratinocytes.

| CELL OR CELL LINE | Kd (nM) | RECEPTORS/CELL |
|------------------|---------|---------------|
| Esophageal Keratinocytes | 16.8 | 400 x 10^3 |
| Esophageal Carcinoma Cell Lines | | |
| HCE-8 | 1.1 | 170 x 10^3 |
| HCE-18 | 0.9 | 100 x 10^3 |
| TE-3 | 0.4 | 92 x 10^3 |
| HCU-39 | 1.2 | 89 x 10^3 |
| TE-2 | 1.1 | 87 x 10^3 |
| TE-1 | 1.0 | 74 x 10^3 |
| HCU-13 | 0.6 | 62 x 10^3 |
| TE-4 | 1.7 | 60 x 10^3 |
| HCE-7(NM) | 0.6 | 52 x 10^3 |
| HCE-9 | 2.7 | 49 x 10^3 |
| HCE-4 | 0.5 | 48 x 10^3 |
| HCE-5 | 0.8 | 39 x 10^3 |
| HCE-6 | 0.3 | 19 x 10^3 |
| HCE-3 | 0.2 | 4.5 x 10^3 |

100-fold higher affinity of binding for EGF, depending on the cell line. Consistent with reports in the literature, we found A431 cells and SCC-15 cells (two epidermoid carcinoma lines) to have elevated numbers of receptors, specifically 2 x 10^8 receptors/cell and 600,000 receptors/cell, respectively (data not shown). These data indicate that the EGF receptor on human esophageal carcinoma cells is biologically different both from that of normal esophageal keratinocytes as well as from other squamous cell carcinomas documented thus far.

Biologic Response of Normal and Transformed Esophageal Epithelial Cells to EGF—To examine for differential effects of EGF on the growth of normal and transformed esophageal keratinocytes, cells were plated at clonal densities (10^5 to 5 x 10^6) onto duplicate 60-mm tissue culture dishes. Initial plating of the cells was carried out in their usual growth medium but depleted of EGF. On the next day, the media was aspirated and fresh medium containing increasing concentrations of EGF in the range of 0 to 40 ng/ml EGF was added to duplicate dishes. When any of the experimental dishes reached approximatly 80% confluence, all of the experimental dishes were fixed and stained with Rhodanile Blue, specific for epithelial cells. The results are shown in Fig. 4. Normal esophageal keratinocytes were maximally stimulated by 10 ng/ml EGF and higher concentrations were inhibitory. Esophageal carcinoma cells, in contrast, while being maximally stimulated by similar concentrations of EGF (namely 5 ng/ml) were not inhibited by higher concentrations up to at least 40 ng/ml. Similar results were obtained with at least 4 other esophageal cell lines tested. The biologic response of these esophageal carcinoma cell lines to EGF stands in stark contrast to that of other squamous cell carcinoma cell lines, such as A431 (shown here) or SCC-15 cells (another epidermoid tumor line) (data not shown), in which the addition of any EGF to the medium, beyond that normally present in serum, was found to markedly inhibit cell growth.

**DISCUSSION**

The role of growth factors and their receptors in transformation is of increasing interest. Because of the importance of
for EGF and its possible altered response to EGF occupancy.

In conclusion, we have shown that elevated EGF receptor levels are not a hallmark of all squamous cell malignancies. Esophageal carcinomas are characterized by fewer receptors with increased ligand affinities compared to normal cells. Moreover, unlike other squamous tumors, they do not exhibit a marked change in their biologic response to EGF compared to normal cells with the exception of their tolerance to high concentrations of EGF.

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