The Epidermal Growth Factor Receptor Is Required to Maintain the Proliferative Population in the Basal Compartment of Epidermal Tumors

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Abstract

Previous studies using keratinocytes from epidermal growth factor receptor (EGFR)-deficient mice revealed that the EGFR is not required for papilloma formation initiated by a mutant ras Ha gene, although the tumors that develop are very small (A. A. Dlugosz et al., Cancer Res., 57: 3180–3188, 1997). The current study used a combination of bromodeoxyuridine pulse-chase, proliferating cell nuclear antigen distribution, and differentiation marker analysis to reveal the following: (a) the EGFR was required to maintain the proliferative population in the basal cell compartment of papillomas; (b) in the absence of EGFR, cycling tumor cells migrated into the suprabasal compartment and initiated the differentiation program prematurely; and (c) these changes were associated with cell cycle arrest. Further analysis of v-ras Ha-transformed EGFR-deficient keratinocytes in vitro indicated that such cells migrated more on and attached less to extracellular matrix components. Together, these studies reveal that an essential function for the EGFR pathway in squamous tumors is to maintain a proliferative pool of basal cells and prevent premature terminal differentiation.

Introduction

The EGFR (1) is proposed to participate in the pathogenesis or maintenance of a number of human cancers of epithelial origin. This supposition is based on the common finding that EGFR ligands are elevated in human tumors or that the EGFR itself is overexpressed, amplified, or constitutively activated by ligand interaction or mutation (reviewed in Ref. 1). Amplification of the EGFR is particularly common in human squamous cell carcinomas (1). The likely participation of the EGFR in epithelial carcinogenesis is supported by a variety of experimental studies as well. Constitutively active EGFR mutants can transform cultured cells (2). Conversely, dominant-negative constructs for EGFR can reverse the transformed phenotype in vitro (3). Transgenic targeting of transforming growth factor α to the mammary gland, skin, and liver enhances tumor formation (4–8). In these models, there is a strong correlation between EGFR and EGFR ligand-induced hyperproliferation and tumorigenesis.

The EGFR pathway appears to be particularly important in skin development and carcinogenesis (reviewed in Ref. 9). Human and mouse squamous cell carcinomas of the skin overexpress EGFR ligands (10–12). Squamous tumors of mouse skin also display constitutive activation of the EGFR kinase function (12). Of particular interest is the profound mouse skin phenotype resulting from genetic deletion of transforming growth factor α or the EGFR. These animals demonstrate premature eye opening, curly hair, aberrant hair follicle differentiation and inflammation, alopecia, and atrophic epidermis (13–15). Similar hair follicle abnormalities were seen in transgenic mice where a dominant-negative EGFR mutant was overexpressed in the epidermis and hair follicles (16). In this case, however, the interfollicular epidermis was paradoxically hyperplastic.

The necessity of the EGFR for skin tumor formation was tested in a model in which primary keratinocytes from EGFR-deficient mice were transformed in vitro by a replication-defective v-ras Ha retrovirus and grafted to a skin site in vivo (17). Epithelial neoplasms (squamous papillomas) formed at the graft site, but they were small, achieving an average size of 20% of similarly treated wild-type keratinocytes (17). Surprisingly, the number of proliferating tumor cells, as measured by BrdUrd incorporation, was high in papillomas of both genotypes (17). Furthermore, there was no increase in apoptotic cells to explain the small size of the EGFR-deficient tumors (17). However, a high percentage of S-phase cells resided in the suprabasal compartment of EGFR-deficient tumors (17). The current study was designed to explain the function of the EGFR in skin tumor development that limits tumor size but does not limit the size of the S-phase pool.

Materials and Methods

Materials. KGF was from R&D Systems (Minneapolis, MN), EMEM was obtained from Life Technologies, Inc. (Rockville, MD), FCS was from Gemini Bio-Products (Calabasas, CA), and penicillin-streptomycin was from Life Technologies, Inc. (Gaithersburg, MD).

Cell Culture and Grafting. Primary keratinocytes were obtained from newborn EGFR-deficient and wild-type mice on a CD-1 background (13). Mice were genotyped using a PCR of tail DNA as described previously (13). Keratinocytes were prepared as described previously (18) and cultured in calcium- and magnesium-free EMEM with 8% chelexed (Bio-Rad Laboratories, Hercules, CA) serum, 20 units/ml penicillin, and 20 μg/ml streptomycin in EMEM adjusted to 0.05 mM calcium using calcium chloride. Cells were initially plated in medium adjusted to 0.25 mM calcium and changed to 0.05 mM calcium-containing medium approximately 18 h later. Cells were cultured in 1 ng/ml KGF to ensure a rapidly proliferating population before infection with a v-ras Ha retrovirus from Ψ-2 producer cell supernatant (19). Viral infection was performed using diluted supernatant from Ψ-2 producer cells in the presence of 4 μg/ml polybrene. KGF was removed from culture media at least 2 days before the start of aggregation, attachment, and Boyden chamber assays. Grafting v-ras Ha-transduced EGFR-deficient and wild-type keratinocytes together with primary dermal fibroblasts onto athymic nude mice, as described previously (17), produced tumors. Fibroblast conditioned medium was produced by incubation of standard medium with SENCAR dermal fibroblasts for 2 days (20). Medium was removed and centrifuged to remove cells and debris before use.

Immunohistochemistry and Immunofluorescence. Tumors were removed from mice after euthanasia, fixed in 70% ethanol, embedded in paraffin, and sectioned. For tissues stained with anti-BrdUrd antibodies, mice were injected with approximately 0.25 mg BrdUrd/g body weight 1 or 24 h before sacrifice. Sections were stained for BrdUrd (Becton Dickinson) or PCNA (Coulter Immunology) as described elsewhere (21). Immunofluorescence experiments using rabbit monospecific antibodies for mouse K1 or K10 (Babco) or mouse monoclonal anti-BrdUrd (Becton Dickinson) were performed as described elsewhere (17, 21).

Attachment Assays. Equal numbers of EGFR-deficient and wild-type keratinocytes were plated in 96-well plates coated with collagen I (Becton Dickinson, Bedford, MA), collagen IV (Becton Dickinson), laminin I (Becton Dickinson), fibronectin (Becton Dickinson), or keratinocyte extracellular matrix. Keratinocyte extracellular matrix was prepared as described in Ref. 22.
Cells were allowed to attach to plates for 1 h, washed twice with PBS, and incubated with fluorescent 4-methylumbelliferylheptanoate (Sigma), and fluorescence was used as a measure of the number of attached cells quantitated using a fluorescent plate reader. In a separate experiment, we confirmed that fluorescence was linearly related to the number of cells attached by also trypsinizing, counting, and plotting the number of cells counted versus fluorescence (data not shown). At least 4 replicate wells/group were used in each experiment. Each experiment was repeated at least once to confirm the results. Data are reported as the mean ± SE. Statistically significant differences between genotypes were determined using Student’s t test.

Cell Migration Assays. Cell migration was measured using Boyden chamber assays. Approximately 100 μl of EMEM (negative control) or 50% EMEM/50% primary dermal fibroblast conditioned medium (experimental group) were placed in the lower chamber of a 48-well Boyden chamber apparatus. Fifty thousand keratinocytes were pipetted into the upper chamber above a 10 μm polyester membrane (Osmonics, Inc.) coated with collagen I, collagen IV, or laminin I. Cells migrating through the membrane were counted using a microscope. At least four microscopic fields for each of the six replicate wells were counted. Each experiment was repeated at least once to confirm the results. Data are reported as the mean ± SE. Statistically significant differences between genotypes were determined using Student’s t test.

Results

Loss of EGFR Results in Altered Cell Cycle Progression in Papillomas. Skin tumors lacking the EGFR have an increase in suprabasal labeling after a 1-h exposure to BrdUrd (17). To determine whether the increase in suprabasal S-phase cells was a consequence of accelerated migration from the basal cell compartment, groups of mice with EGFR-deficient or wild-type graft papillomas were injected with BrdUrd to label S-phase cells and sacrificed 1 or 24 h later. EGFR-deficient papillomas had a significantly higher proportion of S-phase cells in the suprabasal compartment compared with EGFR wild-type control papillomas 1 h after injection (37.4% versus 27.8%, respectively; Table 1; Fig. 1, top panels). Approximately one-sixth of S-phase cells left the basal compartment between 1 and 24 h after injection of BrdUrd in EGFR-deficient papillomas, whereas no decrease in basal S-phase cells was observed in wild-type papillomas at this time point (Table 1). The increase in BrdUrd-labeled basal cells suggests migration of basal cells into the suprabasal cell layers in EGFR-deficient tumors. Total and suprabasal BrdUrd labeling were increased in papillomas at the 24 h time point in both genotypes, consistent with the division of some BrdUrd-labeled cells between 1 and 24 h (Table 1), but the increase in labeled suprabasal cells was greater in wild-type tumors (2.6-fold versus 2.1-fold).

The migration of S-phase cells from basal to suprabasal cell layers, together with a smaller incremental increase in suprabasal BrdUrd-labeled cells after 24 h in EGFR-deficient papillomas, suggested that premature terminal differentiation might cause cell cycle arrest. To examine this possibility, tumor-bearing mice were injected with BrdUrd and sacrificed after 1 h. Tumor specimens were double-stained for BrdUrd and for the differentiation markers K1 and K10 (Fig. 1). Seven times as many BrdUrd-labeled cells expressed K10 in EGFR-deficient papillomas compared with wild-type tumors [44.7% versus 6.3%, respectively (Fig. 1)]. A 3-fold increase in the number of K1-positive S-phase cells was detected in EGFR-deficient tumors compared with wild-type tumors (Fig. 1). Previous results have shown that keratinocytes that express K10 are unable to complete the cell cycle (23, 24). Thus, the increased proportion of S-phase cells expressing K10 represents a loss of a potentially cycling population.

Whereas nuclear BrdUrd incorporation indicates cells passing through S phase, it does not reveal the cell cycle position at the point of examinations. In contrast, subcellular localization and intensity of PCNA staining provide a better indication of cell cycle stage because PCNA is distributed exclusively as intense nuclear or both nuclear and cytoplasmic localization in active S-phase or arrested cells, respectively (25). Immunohistochemical detection of PCNA in EGFR-deficient and wild-type papillomas revealed papillomas lacking the receptor exhibited overall (nuclear plus cytoplasmic) increased suprabasal PCNA labeling (Fig. 1; Table 2). Cytoplasmic PCNA expression was significantly increased, and intensity was decreased in both basal and suprabasal cell compartments, whereas exclusive nuclear PCNA labeling was reduced by 50% in the basal cell compartment of EGFR-deficient papillomas (Table 2). These changes are consistent with a reduction in actively cycling cells in both the basal and suprabasal compartments of EGFR-deficient tumors.

EGFR Status Influences Migration and Attachment of v-rasHa-transduced Keratinocytes. EGFR ligands have been shown to alter keratinocyte migration, attachment, and aggregation in vitro (26, 27). To model the papilloma environment in vitro, keratinocytes from each genotype were transformed by v-rasHa and plated on various matrix substrates in the presence of strontium conditioned medium. Stimulation of migration by fibroblast conditioned medium of v-rasHa-transduced EGFR-deficient keratinocytes through both collagen I- and collagen IV-coated membranes was increased compared with that of wild-type controls in a Boyden chamber assay (Fig. 2). No differences were detected between genotypes in response to serum-free EMEM (Fig. 2). Five h after the start of incubation of keratinocytes in the Boyden chamber, 36% more v-rasHa-transduced EGFR-deficient keratinocytes than EGFR wild-type keratinocytes had migrated through the collagen I-coated membrane (Fig. 2A). Migration of EGFR-deficient keratinocytes through collagen IV-coated membrane was similarly increased by about 60% compared with that of wild-type controls (Fig. 2B).

In vitro attachment of v-rasHa-transduced EGFR-deficient keratinocytes to collagen I-coated, laminin I-coated, and keratinocyte extracellular matrix-coated plates was reduced by 36%, 29%, and 23%, respectively, compared with v-rasHa-transduced wild-type keratinocytes (Fig. 2C). Attachment of EGFR-deficient keratinocytes to collagen IV-coated, fibronectin-coated, and uncoated plates was unaltered (Fig. 2C). Because migration of EGFR-deficient keratinocytes through collagen IV was increased, these data suggest that there are different mechanisms for the alterations in keratinocyte migration and attachment in the absence of EGFR. The decreased attachment and increased migration of EGFR-deficient keratinocytes on basement membrane substrates might contribute to the increased migration of EGFR-deficient S-phase keratinocytes away from the basement membrane in squamous papillomas.

To determine whether EGFR might affect the migration of basal cells to suprabasal cell layers by altering cell-cell aggregation, aggregation of v-rasHa-transduced EGFR-deficient and wild-type keratinocytes was assayed. Previous reports have shown that epidermal

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Time since BrdUrd injection (h)</th>
<th>BrdUrd-labeled nuclei per 100 basal cellsa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BASAL</td>
</tr>
<tr>
<td>EGFR /–/–</td>
<td>1</td>
<td>38.2 ± 2.4</td>
</tr>
<tr>
<td>EGFR +/+</td>
<td>24</td>
<td>41.3 ± 6.9</td>
</tr>
<tr>
<td>EGFR /–/–</td>
<td>24</td>
<td>31.0 ± 2.1</td>
</tr>
<tr>
<td>EGFR +/+</td>
<td>24</td>
<td>41.6 ± 4.4</td>
</tr>
</tbody>
</table>

a The number of BrdUrd-labeled nuclei present in a field of 100 basal cells. 

b Mean is significantly different from that of the corresponding EGFR /–/– time point. 

c Mean is significantly different from 1 h time point, where P = 0.05 using Student’s t test.
Fig. 1. Loss of EGFR resulted in increased migration of S-phase cells from the basal to suprabasal cell compartments and an increased proportion of S-phase keratinocytes that express K1 and K10 in EGFR-deficient papillomas. Grafting of v-ras transduced EGFR wild-type and EGFR-deficient keratinocytes together with primary dermal fibroblasts onto athymic nude mouse hosts produced papillomas. Ethanol-fixed sections of papillomas from mice sacrificed 1 h after injection with BrdUrd were used for immunohistochemistry and immunofluorescence for BrdUrd, K1, K10, and PCNA as described in “Materials and Methods.”

Top panels, immunohistochemistry for BrdUrd with a Harris hematoxylin counterstain.

Middle panels, immunofluorescence for BrdUrd (red), K1 (green), and K10 (green). * value is significantly different from the corresponding EGFR-deficient value (*P ≤ 0.05 using a Student’s t test). Data are given as the mean percentage of BrdUrd-labeled cells that were positive for K1 or K10 ± SE.

Bottom panels, immunohistochemistry for PCNA with a Harris hematoxylin counterstain.

Table 2: Altered subcellular localization of PCNA in EGFR-deficient papillomas

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Basal labeling indexa</th>
<th>Suprabasal labeling index</th>
<th>Total labeling index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nuclear</td>
<td>Cytoplasmic</td>
<td>Nuclear</td>
</tr>
<tr>
<td>EGFR -/-</td>
<td>25.3 ± 6.3</td>
<td>39.7 ± 7.6</td>
<td>19.8 ± 6.6</td>
</tr>
<tr>
<td>EGFR +/-</td>
<td>49.8 ± 6.1b</td>
<td>19.7 ± 5.0b</td>
<td>23.1 ± 5.2</td>
</tr>
</tbody>
</table>

a Labeling index reported as the number of PCNA-labeled cells per 100 basal cells.
b Value was significantly different from that of corresponding EGFR +/+ value (*P ≤ 0.05 using a Student’s t test).
Fig. 2. Increased migration and decreased attachment of v-rastransduced EGFR-deficient keratinocytes in vitro. Migration of v-ras-transduced EGFR-deficient and wild-type keratinocytes through collagen I (A) - and collagen IV (B) -coated membranes was assayed 5 h after the start of incubation in a Boyden chamber. Serum-free EMEM or fibroblast conditioned media (CM) were placed in the lower well of the apparatus. Data are reported as the mean number of cells/field ± SD (A and B). Data are representative of at least two experiments with similar results. C, decreased attachment of v-ras-transduced EGFR-deficient keratinocytes to basement membrane substrates. ECM, keratinocyte extracellular matrix. Data from one experiment representative of at least two performed experiments are expressed as the mean fluorescence ± SE. Equal numbers of v-ras-transduced EGFR-deficient and wild-type keratinocytes were allowed to attach to 96-well plates coated as described in “Materials and Methods.” Fluorescence as a measure of the number of attached cells was quantitated in a plate reader. Attachment is expressed in relative fluorescence units. Relative fluorescence was linearly related to the number of cells attached (data not shown). * mean is significantly different for the value of the corresponding EGFR-deficient value (P ≤ 0.05 using a Student’s t test).

Discussion

Our data indicate that abrogation of EGFR signaling in v-ras-initiated papillomas did not reduce the number of S-phase capable cells but resulted in increased migration of S-phase cells into suprabasal cell compartments and the premature induction of terminal differentiation and cell cycle arrest. These differences were associated with increased migration and decreased basement membrane attachment in culture in v-ras-transduced EGFR-deficient cells. Thus, the decreased papilloma size in the absence of EGFR may be a consequence of the rapid migration of the proliferating pool of initiated basal cells into the differentiating suprabasal compartment. These data suggest that EGFR contributes to tumor growth by maintaining proliferating cells in the basal cell compartment, allowing for subsequent rounds of cell division in these cells.

The phenotype of EGFR-deficient squamous papillomas resembles our prior descriptions of hair follicles from these same mice (21). EGFR-deficient hair follicles that produce wavy and fragile hair are highly proliferative but undergo premature follicular differentiation as detected by expression of keratin 6, transglutaminase, and the hair keratins mHa2 and hacl-1 (21). Thus, EGFR signaling appears to delay commitment to differentiation within the hair follicle as it does in tumors, suggesting that maintaining cells in the proliferative compartment may be a primary function of EGFR in rapidly proliferating tissues. Previous reports have indicated that the activation of EGFR is linked to the differentiation of keratinocytes (15, 21, 28, 29). However, the role of EGFR in keratinocytes is multifaceted because its activation also contributes to cell proliferation in uninitiated keratinocytes (17, 29). Peus et al. (29) found that inhibition of EGFR in human keratinocytes induces expression of K1 and K10. The 3–7-fold increase in BrdUrd-labeled cells that express K1 or K10 in EGFR-deficient tumors is consistent with in vitro studies indicating that paracrine stimulation of keratinocytes through the EGFR negatively regulates expression of these markers (17, 30). Previous reports have shown that ectopic expression of K10 results in the cessation of proliferation in cultured mouse skin papilloma cells (23), delays tumor formation in mouse skin (31), and produces a cell cycle arrest in human keratinocytes that is mediated by the retinoblastoma pathway (24). Activation of EGFR has also been implicated in accelerated late terminal differentiation in human keratinocytes (28). Thus, EGFR could modulate...
cell proliferation in keratinocytes indirectly through the regulation of keratin expression.

Multiple reports document the sometimes contradictory effects of EGF signaling on cell attachment and migration (32–35). Most commonly, EGF activation results in increased cell migration and attachment to basement membrane substrate proteins associated with increased expression of matrix metalloproteinases, particularly matrix metalloproteinase 9 (36–38), and integrins (32–35). These effects are often correlated with increased tumor invasiveness. In contrast, our findings of increased migration and decreased cell attachment in v-rasHa-initiated EGF-deficient keratinocytes were associated with more subtle alterations in tumor migration from basal to differentiating suprabasal cell layers. This behavior is reminiscent of changes in $\alpha_i\beta_j$ and $\beta_i$ integrin expression that are associated with decreased matrix attachment as keratinocytes progress from a stem cell to a transit amplifying phenotype (39, 40), suggesting that the EGF may contribute to this transition. Alternatively, in the absence of EGF signaling, a compensatory up-regulation or activation of receptor signaling pathways known to interact with EGF, such as other EGF family members, or distinct receptor-ligand pathways may be responsible for these effects. EGF ligands are known to interact with other erbB family members in the absence of EGF (41).

In either event, these studies indicate that a principle function of the EGF pathway in squamous tumors is to maintain a proliferative pool of basal cells and prevent premature cell cycle arrest through terminal differentiation.

**References**


11. Rho, O., Beltran, L. M., Gimenez-Conti, I. B., and DiGiovanni, J. Altered expression of matrix metalloproteinases, particularly matrix metalloproteinase 9 (36–38), and integrins (32–35). These effects are often correlated with increased tumor invasiveness. In contrast, our findings of increased migration and decreased cell attachment in v-rasHa-initiated EGF-deficient keratinocytes were associated with more subtle alterations in tumor migration from basal to differentiating suprabasal cell layers. This behavior is reminiscent of changes in $\alpha_i\beta_j$ and $\beta_i$ integrin expression that are associated with decreased matrix attachment as keratinocytes progress from a stem cell to a transit amplifying phenotype (39, 40), suggesting that the EGF may contribute to this transition. Alternatively, in the absence of EGF signaling, a compensatory up-regulation or activation of receptor signaling pathways known to interact with EGF, such as other EGF family members, or distinct receptor-ligand pathways may be responsible for these effects. EGF ligands are known to interact with other erbB family members in the absence of EGF (41).

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