AD_____

Award Number: DAMD17-02-1-0216

TITLE: EGFR-Dependent Regulation of Matrix-Independent Epithelial Cell Survival

PRINCIPAL INVESTIGATOR: Ulrich Rodeck, M.D.

CONTRACTING ORGANIZATION: Thomas Jefferson University
Philadelphia, PA 19107

REPORT DATE: April 2007

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-04-2007	Final Addendum	15 Mar 2006 – 14 Mar 2007

4. TITLE AND SUBTITLE

EGFR-Dependent Regulation of Matrix-Independent Epithelial Cell Survival

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-02-1-0216

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Ulrich Rodeck, M.D.

E-Mail: ulrich.rodeck@mail.tju.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Thomas Jefferson University
Philadelphia, PA 19107

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Background: Signaling through the epidermal growth factor (EGFR) has been implicated in both effective wound healing and epithelial neoplasia. We have identified a novel function of the EGFR in support of epithelial cell survival, particularly in conditions of anchorage-independence.

Objective/Hypothesis: Define molecular mechanisms and pathways by which EGFR activation supports epithelial cell survival. Two specific aims focus on (1) posttranslational modification of relevant Bcl-2 family members by EGFR activation through MAPK-dependent mechanisms and, (2) STAT3 activation by deregulated EGFR signaling as observed in epithelial cancer.

Progress: Work related to Specific Aim 1 has been completed and published in three manuscripts during 2006. In addition, another manuscript has been accepted for publication in 2007. The final action item, i.e. assessment of JNK and p38 activation in the anchorage-independent state as they relate to NF-kappaB activity has been completed. In summary, we have completed the work proposed in both, Specific Aims 1 and 2 of the original proposal.

15. SUBJECT TERMS
receptor; epidermal growth factor; cell survival; cell transformation; neoplastic; mitogen-activated protein kinases

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	58	**19b. TELEPHONE NUMBER** *(include area code)*

TABLE OF CONTENTS

INTRODUCTION

The epidermal growth factor receptor (EGFR) is a type-1 receptor tyrosine kinase. Activation of the EGFR has been implicated in many aspects of cell biology pertaining to wound healing and malignant transformation of epithelial cells. Previous work from our laboratory underscored a novel function of the EGFR in support of cell survival of epidermal keratinocytes. Specifically, EGFR activation and signaling was observed to protect keratinocytes against induction of apoptosis through extracellular stressors (1, 2). The major thrust of the research funded through **DAMD17-02-1-0216** was to further characterize the protective role of EGFR activation in situations in which keratinocytes encounter suboptimal extracellular matrix interaction, i.e. during wound healing and metastatic spread of malignant cells. We originally proposed to pursue two specific aspects of EGFR activation as they relate to cell survival. Specific Aim 1 focused on posttranslational modifications of regulators of cell survival of the Bcl-2 family of molecules through EGFR activation and suspension culture. Specific Aim 2 dealt with the question whether inappropriate signaling through the EGFR as observed in cancer cells leads to aberrant STAT3 activation, which in turn enhances cell survival.

BODY

This progress report represents an addendum to the final progress report submitted last year. As required it contains a summary of the research accomplishments during the whole period of support through **DAMD17-02-1-0216**. As such it necessarily duplicates some sections previously submitted. The accomplishments are listed relative to the Specific Aims of the original proposal.

However, we would like to start by summarizing research performed during the last 12 months, i.e. during the period of no-cost extension of this grant. We had requested a no-cost extension to complete work on signaling crosstalk as it relates to NF-κB and JNK/p38MAPkinase signaling in immortalized keratinocytes subjected to anchorage-independence. Specifically, we focused on MAPkinases JNK and p38 in the anchorage-independent state. We noticed strong JNK/p38 phosphorylation in suspension culture, which was only marginally affected by EGFR activation. We decided to evaluate the relevance of these two MAPKs to keratinocyte death in forced suspension culture based on previous reports that they exert pro- or anti-apoptotic effects in different cell systems and experimental settings (3-7). We observed that pharmacological inhibition of JNK but not p38 moderately attenuates apoptosis of keratinocytes in the anchorage-independent state. We are now investigated the role of NF-κB activation in counteracting pro-apoptotic JNK and/or p38 activity in the anchorage-independent state. This was based on previous reports that sustained JNK activation contributes to apoptosis when NF-κB activation is blocked (8, 9). Collectively, these previous studies highlighted complex roles and regulation of MAPkinases in keratinocyte apoptosis resistance and formed the basis for the hypothesis that EGFR-dependent MAPK activation protects keratinocytes whereas JNK and p38 activation may exert pro-apoptotic effects which might be counteracted by NF-κB activation. We have addressed the functional roles of JNK/MAPKinase signaling in forced suspension cultures using pharmacological

inhibitors of these pathways. The results revealed that inhibition of JNK/p38 does not markedly affect the cell survival or death by apoptosis in the anchorage-independent state. This applies not only to non-transfected cells but also to immortalized cells engineered to overexpress the NF-kBp65 subunit.

Specific Aim 1

We have published the manuscript ***'EGFR-dependent downregulation of Bim in epithelial cells requires MAPK and PKC-delta activities' by Quadros, M.A., Connelly, S., Kari, C., Abrams, M.A., Wickstrom, E., and Rodeck. U. Cancer Biology and Therapy 5:498-504, 2006***. In this work we evaluated posttranslational modification of the proapoptotic Bcl-2 family member Bim during suspension culture and in the presence and absence of EGFR activation in HaCaT keratinocytes. The results can be summarized as follows:

- Activation of the epidermal growth factor receptor (EGFR) provides a measure of protection to immortalized epidermal keratinocytes (HaCaT cells) against apoptosis induced by diverse cellular stressors. This effect is due, in part, to sustained MAPK-dependent Bcl-xL expression.
- We identified a second EGFR/MAPK-dependent signaling event that protects HaCaT cells against apoptosis incurred during forced suspension culture (anoikis). This pathway targets Bim, a pro-apoptotic BH3-only Bcl-2 family member.
- Bim expression was functionally relevant to HaCaT cell survival as demonstrated by partial protection against anoikis provided by siRNA-induced Bim downregulation (see Fig. 1 in manuscript #1 appended).
- Growth factor starvation of appended and suspended cells was associated with enhanced Bim expression whereas EGFR activation reduced Bim expression by inducing Bim phosphorylation and proteasomal degradation (see Figs. 2 and 4 in manuscript #1 appended).
- EGFR-dependent Bim phosphorylation required MAPK activation. Furthermore, PKC-δ activity contributed to both MEK/MAPK phosphorylation and Bim phosphorylation as demonstrated using both pharmacological inhibitors of PKC-δ and siRNA-mediated PKC-δ knockdown (see Fig. 5 in manuscript #1 appended)..
- In addition to HaCaT cells, EGFR activation supported survival and induced Bim phosphorylation in several squamous carcinoma cell lines in a strictly MAPK-dependent fashion (see Fig. 6 and 7 in manuscript #1 appended)..
- These results establish that EGFR activation attenuates susceptibility of immortalized and malignant keratinocytes to apoptosis by posttranslational control of Bim-EL expression through a pathway requiring PKC-δ and MEK/MAPK activation.

A second manuscript was also published: ***'A novel role for E-cadherin loss during initiation of squamous cell carcinoma through modulation of integrin-mediated matrix adhesion' by Zhang, W., Alt-Holland, A., Margulis, A., Rodeck, U., Fusenig, N.E., and Garlick, J.A. J. Cell Science 119:283-92, 2006.*** The following results were

described:

- Novel 3D human tissue constructs that mimic premalignant disease in normal epidermis were used to directly investigate how loss of E-cadherin directs conversion to malignant disease.
- A genetically tagged variant of Ha-Ras-transformed human keratinocytes expressing dominant-interfering (DI) E-cadherin was used.
- DI-E-cadherin expressing cells persisted at the basement membrane zone of skin reconstructs enabling tumor cell invasion upon transplantation to mice ((see Figs. 1 – 3 in manuscript #2 appended).
- DI-E-cadherin cells expressed increased levels of alpha2, alpha3, and beta1 integrins and increased adhesion to basement membrane components laminin-1 and types I and IV collagen (see Figs. 4 and 5 in manuscript #2 appended).
- These results establish a novel aspect of E-cadherin function as it relates to cell-matrix adhesion.

A third manuscript with direct relevance to the survival phenotype of human keratinocytes in the anchorage-independent state was published: '***Malignant transformation of immortalized HaCaT keratinocytes through deregulated NF-κB signaling' by Ren, Q., Kari, C., Quadros, M.R., Burd, R., McCue, P., Dicker, A.P., and Rodeck, U. Cancer Res. 66: 5209-15.*** This work expands the scope of cell survival mechanisms in epidermal keratinocytes beyond the EGF receptor. Specifically, it provides evidence that:

- specific matrix components protected human immortalized keratinocytes (HaCaT) against apoptosis induced by blockade of either the epidermal growth factor receptor (EGFR) or MEK or PI3-kinase (see Fig. 3 in manuscript #3 appended).
- In contrast, nuclear factor NFκB activation was indispensable to HaCaT cell survival regardless of matrix availability (see Figs. 1 and 2 in manuscript #3 appended).
- Conversely, tumor necrosis factor (TNF)-α supported anchorage-independent survival of HaCaT cells in an NFκB-dependent fashion (see Fig. 5 in manuscript #3 appended).
- Deregulated NF-κB signaling by overexpression of the NF-κB p65 subunit converts immortalized HaCaT keratinocytes to fully malignant, tumorigenic cells (see Fig. 6 in manuscript #3 appended).

These results highlight previously unrecognized patterns of signal integration between survival pathways relevant to wound healing and malignant transformation of the skin in that NF-κB signaling and EGFR signaling cooperatively induce anchorage-independent keratinocyte survival. This work has direct translational relevance as upregulation of NF-κB signaling in wound healing may provide a second therapeutic approach in addition to stimulating EGFR activation. This work further provided the basis for a grant application to NIH to further dissect NF-κB signaling in keratinocyte survival with a particular focus

on the interplay of NF-κB signaling with JNK/p38MAPKinases (see above).

A fourth manuscript was published in 2004 (Manuscript #4 appended). This paper was an invited book chapter to describe the experimental details of the system we have used throughout the studies performed. It represents a technical report which was peer-reviewed.

Specific Aim 2

As outlined in the previous progress reports we established that EGFR-dependent STAT3 activation is relevant to growth and survival of malignant but not untransformed normal keratinocytes or partially transformed HaCaT cells (see manuscript # 5 in reportable outcomes and appended). In this paper we demonstrated that:

- STAT3 activation as assessed by Y705 phosphorylation is prevalent in squamous cell carcinomas but not normal keratinocyte cultures (Fig. 2 in manuscript # 5 appended);

- STAT3 phosphorylation in SCC is only partially due to EGFR activation (Figs. 2 and 3 in manuscript # 5 appended);

- Forced EGFR expression in immortalized is not sufficient to trigger STAT3 activation in immortalized HaCaT keratinocytes (Fig. 5 in manuscript # 5 appended);

- Upon EGF stimulation STAT3 phosphorylation is negatively regulated by MEK/MAPK signaling in immortalized keratinocytes (Fig. 6 in manuscript # 5 appended);

Dr. Rodeck has participated in two PRMRP meeting for DOD grantees in April 2004 and in April 2006 in San Juan, Puerto Rico. On both occasions Dr. Rodeck presented posters describing the research. In 2004, results subsequently oublished in manuscript #5 were presented. In 2006, the presentation focused on the characterization of a novel radiation protector based on the nanoparticle DF-1 (C-Sixty Inc.) in a novel *in vivo* vertebrate systen (zebrafish embryos). This work has been since been published in Clinical Cancer Research (***Daroczi, B., Kari, G., McAleer, M.F., Rodeck, U., and Dicker, A.P. (2006) In vivo radioprotection by the carboxyfullerene DF-1 as assessed in a zebrafish model. Clin Cancer Res. 12:7086-91***). Although not directly supported by DAMD17-02-1-02156 this work has military relevance to prevent radiation-induced morbidity and mortality in the war fighter as well as the civilian population.

The following personnel was supported through **DAMD17-02-1-0216** for the time periods indicated:

Rodeck, Ulrich	03/09/02-04/13/07
Kari, Csaba	03/09/02-04/31/07

Connelly, Sharon	08/24/02-03/18/06
Huggett, Teresa	03/09/02-07/11/03
DeQuadros, Marlene	09/21/02-04/28/06
Orlova, Irina	03/08/03-10/01/04

KEY RESEARCH ACCOMPLISHMENTS

Specific Aim 1

- STAT3 activation is a tumor-associated phenomenon in cultured keratinocytes and squamous cell carcinomas.
- Certain SCC carcinomas exhibit 'constitutive' STAT3 activation independently of EGFR activation.

Specific Aim 2

- The pro-apoptotic Bcl-2 family member Bim is a target of the EGFR/MAPK signaling axis in normal and transformed keratinocytes;
- Regulation of the proapoptotic Bcl-2 family member Bim occurs by posttranslational modification (phosphorylation) targeting Bim for proteasomal degradation;
- Identification of alpha2, alpha3, and beta1 integrins as targets for E-cadherin signaling with implications for enhanced matrix interaction and tumorigenicity;
- Characterization of a role of NF-κB activation in support of inappropriate keratinocyte survival in the absence of extracellular matrix interaction;
- Demonstration that, in keratinocytes, NF-κB signaling occurs in an extracellular matrix attachment-dependent but EGFR-independent fashion;
- Demonstration that NF-κB and EGFR signaling occur independently to support anchorage-independent survival of immortalized keratinocytes;
- Pro-apoptotic signals by JNK and p38 activation in the anchorage-independent state are not significantly counteracted by NF-κB activity.

Collectively, these findings support the concept of complex regulation of enhanced keratinocyte survival during wound healing and cancer in which the EGFR plays a major role which is complemented by alterations of cell-cell adhesion molecules (E-cadherin) and of cell-matrix adhesion which supports the NF-κB pathway. The relevance of these findings relates to the design of combination therapies targeting the EGFR as well as NF-κB to disrupt skin tumorigenesis. Conversely, pharmacological EGFR activation and stimulation of NF-κB activation could be considered in the context of acceleration of wound healing.

REPORTABLE OUTCOMES

During the funding period six peer-reviewed manuscripts supported by **DAMD17-02-1-0216** were published. In addition, during the last year one manuscript (#6) was accepted for publication, which addresses the consequences of EGFR activation on transcriptional profiles of malignant keratinocytes in squamous cell carcinomas in situ.

Manuscript #1:

Quadros, M.A., Connelly, S., Kari, C., Abrams, M.A., Wickstrom, E., and Rodeck. U EGFR-dependent downregulation of Bim in epithelial cells requires MAPK and PKC-activities. Cancer Biology and Therapy 5:498-504, 2006.

Manuscript #2:

Zhang, W., Alt-Holland, A., Margulis, A., Rodeck, U., Fusenig, N.E., and Garlick, J.A A novel role for E-cadherin loss during initiation of squamous cell carcinoma through modulation of integrin-mediated matrix adhesion. J. Cell Science 119:283-92, 2006

Manuscript #3:

Ren, Q., Kari, C., Quadros, M.R., Burd, R., McCue, P., Dicker, A.P., and Rodeck, U. Malignant transformation of immortalized HaCaT keratinocytes through deregulated NF-κB signaling. Cancer Res. 66: 5209-15, 2006.

Manuscript #4:

Jost, M., and Rodeck, U. 2004. Keratinocyte culture in the absence of substrate attachment. In: Methods in Molecular Biology Vol. 289:23-28. (K. Turksen, ed.). Humana Press.

Manuscript #5:

Quadros, M.R., Peruzzi, F., Kari, C., and Rodeck, U. 2004. Complex regulation of signal transducer and activator of transcription 3 activation in normal and malignant keratinocytes. Cancer Res. 64:3934-3939.

Manuscript #6:

Chung, C.H., Parker, J., Levy, S., Slebos, R. J., Dicker, A. P. and Rodeck, U. 2007 Gene expression profiles as markers of aggressive disease - EGFR as a factor. Int J Radiat Oncol Biol Phys. (in press).

In addition to these original manuscripts, three review articles focusing on extracellular matrix control of epidermal malignancy were published or accepted for publication during the funding period:

Review #1:

Kari, C., Chan, T.O., Rocha de Quadros, M., and Rodeck, U. 2003. Targeting the Epidermal Growth Factor Receptor in cancer: Apoptosis takes center stage. Cancer Res. 63:1-5

Review #2:

Dicker A.P., and Rodeck, U. 2005. Predicting the future from trials of the past: epidermal growth factor receptor expression and outcome of fractionated radiation therapy trials. Journal of Clinical Oncology. 23:5437-9.

Review #3:

Rodeck, U., Fertala, A., and Uitto, J. 2007. Anchorless keratinocyte survival - An emerging pathogenic mechanism for squamous cell carcinoma in recessive dystrophic eepidermolysis bullosa. Experimental Dermatology 16:465-7.

Review #4:

Rodeck, U. and Uitto, J. 2007. RDEB-associated SCC – an enigmatic entity with complex pathogenesis. Journal of Investigative Dermatology (in press).

PDFs of the published manuscripts are appended to this report (see Appendix section below) and contain multiple visual representations of the results described above and referenced in the Body section as appropriate.

CONCLUSIONS

During funding period we have accomplished the objectives laid out in Specific Aims 1and 2 of the original proposal. The results obtained have identified key players that coordinate keratinocyte survival dependent on soluble growth factors of the EGF family, cell matrix adhesion receptors of the integrin family and cell-cell adhesion receptors of the cadherin family. They highlighted complex, partially redundant regulatory mechanisms in support of keratinocyte survival during the wound healing process and in advanced stages of malignancy.

REFERENCES

1. Kari C, Chan TO, Rocha de Quadros M, Rodeck U. Targeting the epidermal growth factor receptor in cancer: apoptosis takes center stage. Cancer Research 2003;63(1):1-5.
2. Rodeck U, Jost M, Kari C*, et al*. EGF-R dependent regulation of keratinocyte survival. Journal of Cell Science 1997;110(Pt 2):113-21.
3. Fuchs SY, Adler V, Pincus MR, Ronai Z. MEKK1/JNK signaling stabilizes and activates p53. Proceedings of the National Academy of Sciences of the United States of America 1998;95(18):10541-6.
4. Cardone MH, Salvesen GS, Widmann C, Johnson G, Frisch SM. The regulation of anoikis: MEKK-1 activation requires cleavage by caspases. Cell 1997;90(2):315-23.
5. Antonyak MA, Moscatello DK, Wong AJ. Constitutive activation of c-Jun N-terminal kinase by a mutant epidermal growth factor receptor. Journal of Biological Chemistry 1998;273(5):2817-22.
6. Smith A, Ramos-Morales F, Ashworth A, Collins M. A role for JNK/SAPK in proliferation, but not apoptosis, of IL-3-dependent cells. Current Biology 1997;7(11):893-6.
7. Assefa Z, Vantieghem A, Declercq W*, et al*. The activation of the c-Jun N-terminal kinase and p38 mitogen-activated protein kinase signaling pathways protects HeLa cells from apoptosis following photodynamic therapy with hypericin. Journal of Biological Chemistry 1999;274(13):8788-96.
8. Tang G, Minemoto Y, Dibling B*, et al*. Inhibition of JNK activation through NF-kappaB target genes. Nature 2001;414(6861):313-7.
9. Tang F, Tang G, Xiang J, Dai Q, Rosner MR, Lin A. The absence of NF-kappaB-mediated inhibition of c-Jun N-terminal kinase activation contributes to tumor necrosis factor alpha-induced apoptosis. Molecular & Cellular Biology 2002;22(24):8571-9.

APPENDIX

This Appendix consists of PDF files of published manuscripts assembled in the following order:

1. Quadros et al., Cancer Biology and Treatment, 2006
2. Zhang et al., Journal of Cell Science, 2006
3. Ren et al., Cancer Research, 2006
4. Jost and Rodeck, Methods in Molecular Biology Vol. 289, 2004
5. Quadros et al., Cancer Research, 2004
6. Kari et al., Cancer Research, 2003
7. Rodeck et al., Experimental Dermatology, 2007
8. Dicker and Rodeck, Journal of Clinical Oncology, 2005

[Cancer Biology & Therapy 5:5, 498-504, May 2006]; ©2006 Landes Bioscience

Research Paper

EGFR-Dependent Downregulation of Bim in Epithelial Cells Requires MAPK and PKC-δ Activities

Marlene R. Quadros[1]
Sharon Connelly[1]
Csaba Kari[1]
Marc T. Abrams[2]
Eric Wickstrom[2]
Ulrich Rodeck[1,*]

Departments of [1]Dermatology and Cutaneous Biology and [2]Biochemistry and Molecular Pharmacology; Thomas Jefferson University; Philadelphia, Pennsylvania USA

*Correspondence to: Ulrich Rodeck; Department of Dermatology and Cutaneous Biology; Thomas Jefferson University; 233 S. 10th Street; BLSB 319; Philadelphia, Pennsylvania 19107 USA; Tel.: 215.503.5622; Fax: 215.503.5622; Email: Ulrich.Rodeck@mail.tju.edu

Received 10/18/05; Accepted 02/06/06

Previously published online as a *Cancer Biology & Therapy* E-publication: http://www.landesbioscience.com/journals/cbt/abstract.php?id=2567

KEY WORDS

Bim, EGFR, apoptosis, anoikis, PKC-δ, MAPK, keratinocytes, squamous cell carcinoma

ABBREVIATIONS

EGFR	epidermal growth factor
MAPK	mitogen activated protein kinase
PKC-δ	protein kinase C-delta
IGFR1	insulin-like growth factor receptor 1
PI-3 kinase	phosphatidylinositol 3-kinase
MEK	mitogen activated protein kinase kinase
ILK	integrin linked kinase
FAK	focal adhesion kinase

ACKNOWLEDGEMENTS

We thank Dr. N. Fusenig and Dr. J. Rheinwald for providing cell lines. This work was supported by grants from NIH-NCI (CA81008 to U.R.), the Department of Defense (DAMD17-02-1-0216 to U.R.) and the Department of Energy (63055 to E.W.).

ABSTRACT

Activation of the epidermal growth factor receptor (EGFR) provides a measure of protection to immortalized epidermal keratinocytes (HaCaT cells) against apoptosis induced by diverse cellular stressors. This effect is due, in part, to sustained MAPK-dependent Bcl-x_L expression. Here, we report a second EGFR/MAPK-dependent signaling event that protects HaCaT cells against apoptosis incurred during forced suspension culture (anoikis). This pathway targets Bim, a pro-apoptotic BH3-only Bcl-2 family member. Bim expression was functionally relevant to HaCaT cell survival as demonstrated by partial protection against anoikis provided by siRNA-induced Bim downregulation. Growth factor starvation of attached and suspended cells was associated with enhanced Bim expression whereas EGFR activation reduced Bim expression by inducing Bim phosphorylation and proteasomal degradation. EGFR-dependent Bim phosphorylation required MAPK activation. Furthermore, PKC-δ activity contributed to both MEK/MAPK phosphorylation and Bim phosphorylation as demonstrated using both pharmacological inhibitors of PKC-δ and siRNA-mediated PKC-δ knockdown. In addition to HaCaT cells, EGFR activation supported survival and induced Bim phosphorylation in several squamous carcinoma cell lines in a strictly MAPK-dependent fashion. These results establish that EGFR activation attenuates susceptibility of immortalized and malignant keratinocytes to apoptosis by post-translational control of Bim-EL expression through a pathway requiring PKC-δ and MEK/MAPK activation.

INTRODUCTION

Environmental cues constitute an important organizing principle in multicellular organisms not only during embryogenesis but throughout life. In recent years it has become obvious that external control of cellular physiology entails at least three types of signals, i.e., soluble factors such as hormones, growth factors or cytokines, activation of cell/cell adhesion receptors and, adhesion to extracellular matrix (ECM). The crucial importance of ECM in maintaining epithelial cell physiology is underlined by the fact that such cells will die by apoptosis when matrix attachment is denied in a process named 'anoikis'.[1] This mechanism is thought to avert dissemination of epithelial cells to inappropriate sites, most notably during the metastatic process. This concept has led several groups of investigators to study mechanisms by which epithelial cells may avoid apoptosis upon losing contact with their physiological matrix. Different mechanisms have been described including enhanced expression and function of elements of the signal transduction machinery usually activated by integrins, i.e., Fak[2] and Ilk.[3,4] Perhaps not surprisingly, receptor tyrosine kinases with protooncogenic properties have similarly been implicated in resistance to anoikis. Two well-studied examples in this class of anoikis inhibitors are the receptor for insulin-like growth factor1 (IGFR1)[5] and the epidermal growth factor receptor (EGFR).[6] Both receptors have been shown to affect the balance of members of the Bcl-2 family of apoptosis regulators in favor of an anti-apoptotic phenotype. For example, EGFR activation supports expression of the anti-apoptotic Bcl-x_L[6,7] and has been reported in certain cell types to cause phosphorylation of pro-apoptotic Bad associated with functional inactivation of its pro-apoptotic properties.[8]

Recently, another pro-apoptotic Bcl-2 family member (Bim) has been implicated in regulation of cell death in several cell types. Bim was initially identified as a Bcl-2 binding protein.[9] Subsequent studies showed that Bim, a BH3-only Bcl-2 relative, plays an essential pro-apoptotic role in activation-induced T lymphocyte death.[10,11] Mice lacking Bim expression accumulate immune cells of various lineages associated with the late development of autoimmune disorders. Reginato et al.[12] recently showed that forced suspension culture

of immortalized mammary epithelial cells is associated with increased Bim expression that can be counteracted by EGF treatment. Furthermore, siRNA-mediated Bim downregulation leads to partial rescue of these cells from anoikis highlighting the functional relevance of Bim in this process.

Here we describe that Bim expression in immortalized human keratinocytes (HaCaT cells) is similarly regulated by EGFR activation and is functionally relevant to cell survival in the absence of matrix attachment. Specifically, we observed that reduction of Bim expression by siRNA partially protects HaCaT keratinocytes form cell death in suspension culture. Furthermore, we describe a molecular mechanism by which EGFR activation counteracts Bim expression and proapoptotic function. Specifically, in attached and suspended epidermal cells, EGFR activation downregulates Bim expression by inducing Bim phosphorylation and proteasomal degradation. This phenomenon rested on EGFR-dependent MEK/MAPK activation. Furthermore, we demonstrate that PKC-δ activity is a necessary prerequisite for MAPK-dependent Bim phosphorylation and acts upstream of MEK/MAPK activation.

MATERIALS AND METHODS

Materials. Properties of the EGFR antagonistic mAb 425 have been described earlier.[13,14] The MEK inhibitors PD 98059 and U0126, PI3-kinase inhibitor LY294002, the JAK-2 inhibitor AG 490, the JNK inhibitor I, the p38 MAPkinase inhibitor SB203580 and the AG1478 tyrphostin were purchased from Calbiochem-Novabiochem (San Diego, CA). The Src inhibitor PP1 and the PLCγ inhibitor U73122 were purchased from either Calbiochem or Biomol (Plymouth Meeting, PA). The PKCα/β inhibitor Gö6976 and the PKCδ inhibitor rottlerin were purchased from Biomol. Antibodies to Bim and Bad were purchased from Calbiochem. Antibodies to PKCδ were purchased from Santa Cruz Laboratories (Santa Cruz, CA), and antibodies to α-tubulin from Oncogene (LaJolla, CA). Antibodies to p42/44 MAPK, phospho-MAPK and phospho-MEK1/2 were purchased from Cell Signaling Technology (Beverly, MA) or Santa Cruz Biotechnology. Hemagglutinin tag antibodies were purchased from Covance (Richmond, CA).

Cell lines. Primary human keratinocytes, immortalized HaCaT keratinocytes and squamous carcinoma cell lines SCC 9, SCC 12, FaDu and, A431 were maintained in culture as described earlier.[15,16] SCC 9, SCC 12 and, FaDu cells were kindly provided by Dr. James Rheinwald, Boston, MA, and HaCaT cells conditionally expressing constitutively active MEK-1 (pCEPTetP-GIC-MKK1) were described earlier.[17]

Cell culture conditions and forced suspension culture. For treatment with inhibitors, cells were seeded at subconfluency in a serum-free medium formulated for keratinocyte culture (Keratinocyte-SFM; GIBCOBRL, Carlsbad, CA). After attachment, medium was replaced with Keratinocyte-SFM (supplemented with 0.2% BSA-FAF) that lacked any protein growth factors (base medium) unless noted otherwise for at least 12 h. Two hours prior addition of EGF, inhibitors were added directly into the culture medium. EGF was then added for 15 min and cells were harvested for analysis as described below. Forced suspension culture was performed using protein-free Keratinocyte-SFM as described before.[17] Rescue after forced suspension was performed by reseeding cells either into serum-containing routine culture medium for HaCaT or squamous carcinoma cells or fully complemented Keratinocyte-SFM for normal keratinocytes.

SiRNA treatments. Purified siRNA duplexes (siRNAs) were purchased from Dharmacon (Lafayette, CO). The siRNA sequences for targeting Bim were *Bim* siRNA1 (5'-AAU UGU CUA CCU UCU CGG U dTdT-3') and *Bim* siRNA 2 (5'- UCU GUC UGU AGG GAG GUA G dTdT-3'). The siRNA sequences for targeting PKCδ were *PKC*δ siRNA 1 (5'- GAU GAA GGA GGC GCU CAG dTdT-3') and *PKC*δ siRNA 2 (5'- GGC UGA GUU CUG GCU GGA C dTdT- 3'). Control cultures were transfected with either siRNA targeting firefly luciferase (Dharmacon) or silencer negative control#1siRNA (Ambion, Austin, TX). HaCaT cells were seeded in 6-well plates or 100 mm dishes at low confluency (~50%) one day before transfection. Cultures were transfected with 200 nM double-stranded siRNA using RNAifect (Qiagen, Valencia, CA). Specifically, a 20 μM stock solution of double-strand siRNA was diluted with serum-free medium and mixed with 15 μl of RNAifect at a ratio of siRNA to liposome of 2:3 v/v. Batch dilutions of liposomes were performed for each 6-well plate and preincubated at room temperature for 15 min before addition to the medium-diluted siRNA. For standard assays of activity, cells were harvested at 24, 48 and 72 hours after transfection.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was prepared using Trizol and reverse transcribed as described previously[18] using oligo(dT) primers and AMV reverse transcriptase. Oligonucleotide primers corresponding to sequences flanking the initiation and termination codons of *Bim* were used (forward primer ATGGCAAAGCAACCT; reverse primer TCAATGCATTCTCCA). These primers amplify all known *Bim* isoforms generated by alternative splicing.[19] In addition, a *Bim-EL* specific reverse primer was used to amplify *Bim-EL* sequences only (GTGCTGG-GCTCCTGT). This primer covers the exon 3-4 junction present only in *Bim-EL* transcripts and produces a 320 bp amplimer when used with the forward primer specified above. PCR was performed using the following temperature profile: 94°C-58°C-72°C for 1 min each and 35 cycles. PCR was terminated using a 7 min extension period at 72°C. RT-PCR products were analyzed on a 2%-agarose gel with 100 bp ladder markers (Roche Diagnostics, Indianapolis, IN). PCR products corresponding in size to BIM-EL, -L, and -S were reamplified, purified and subjected to diagnostic restriction enzyme analysis using Pst, Apo and PvuII, respectively.

Western blot analysis. Cells were lysed in Laemmli buffer followed by boiling for 3–5 min. Equal amounts of protein were separated by SDS-PAGE and blotted onto PVDF or nitrocellulose membranes (Millipore). Membranes were blocked in blocking buffer (5% dry milk in TBS) and then incubated with dilutions of primary antibodies in antibody buffer (5% BSA, 0.05% Tween 20 (Sigma Aldrich, St. Louis, MO) in TBS) followed by incubation in dilutions of HRP-conjugated secondary antibodies in the same buffers. Following antibody incubations, membranes were washed in TTBS (0.5% Tween 20 in TBS). Signals were visualized by chemiluminescence using reagents from Pierce Chemical Co. (Rockford, IL) according to the manufaturer's instructions. After detection, blots were washed and stripped using Restore™ Western Blot Stripping Buffer (Pierce) and used for further antibody incubations.

RESULTS AND DISCUSSION

Bim expression contributes to anoikis in HaCaT keratinocytes. A recent report described that lack of extracellular matrix interaction induces expression of the pro-apoptotic Bcl-2 family member Bim in MCF-10A mammary epithelial cells.[12] This effect contributes to anoikis, i.e., death of MCF-10A cells in the absence of matrix attachment, and is counteracted by EGFR activation. To gauge the relative contribution of Bim expression to anoikis sensitivity of immortalized human keratinocytes (HaCaT cells) we compared survival of these cells in forced suspension culture upon downregulation of Bim expression using RNA interference. To downregulate Bim expression Bim mRNA was targeted using siRNA duplexes with maximal effect on Bim protein expression within 24 h after transfection (Fig. 1A). To allow for optimal siRNA effect, subsequent experiments were initiated 48 h after transfection of Bim siRNA. To ascertain cell survival after suspension culture, aliquots of suspended cells were reseeded on cell culture-treated plastic and the fraction of reattached, viable cells was assessed spectroscopically upon staining with crystal violet 24 h after reseeding. This analysis showed that Bim siRNA treatment was associated with partial rescue of HaCaT cells maintained in suspension culture in the presence of either the MEK inhibitor

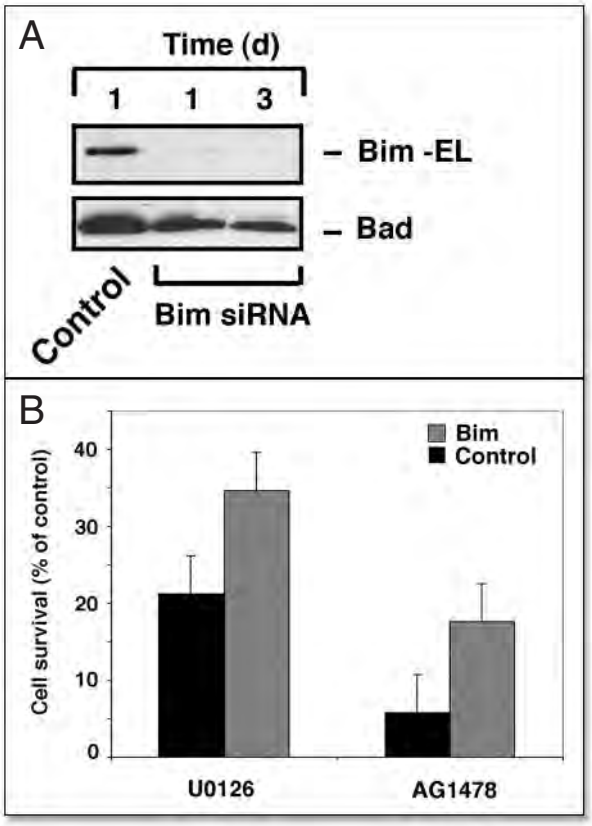

Figure 1. Expression of Bim-EL in HaCaT keratinocytes and effect of Bim downregulation on survival of HaCaT cells in forced suspension cultures. (A) Bim-EL expression in HaCaT cells transfected with siRNA targeting *Bim* RNA. Effects on Bim protein expression were apparent after 24 h of transfection. (B) Improved survival of HaCaT cells in forced suspension culture after *Bim* siRNA transfection. Forty-eight hours after transfection of either *Bim* or control siRNA, cells were placed in suspension culture for 48 h in the presence of EGF with either the EGFR inhibitorAG 1478 (1 μM) or the MEK inhibitor U0126 (10 μM) as indicated. Survival was assessed by spectrophotometric measurement of crystal violet staining of cells replated on tissue culture-treated plastic after 24 h of forced suspension culture. Staining of EGF-treated cultures in the absence of inhibitors was designated as 100%.

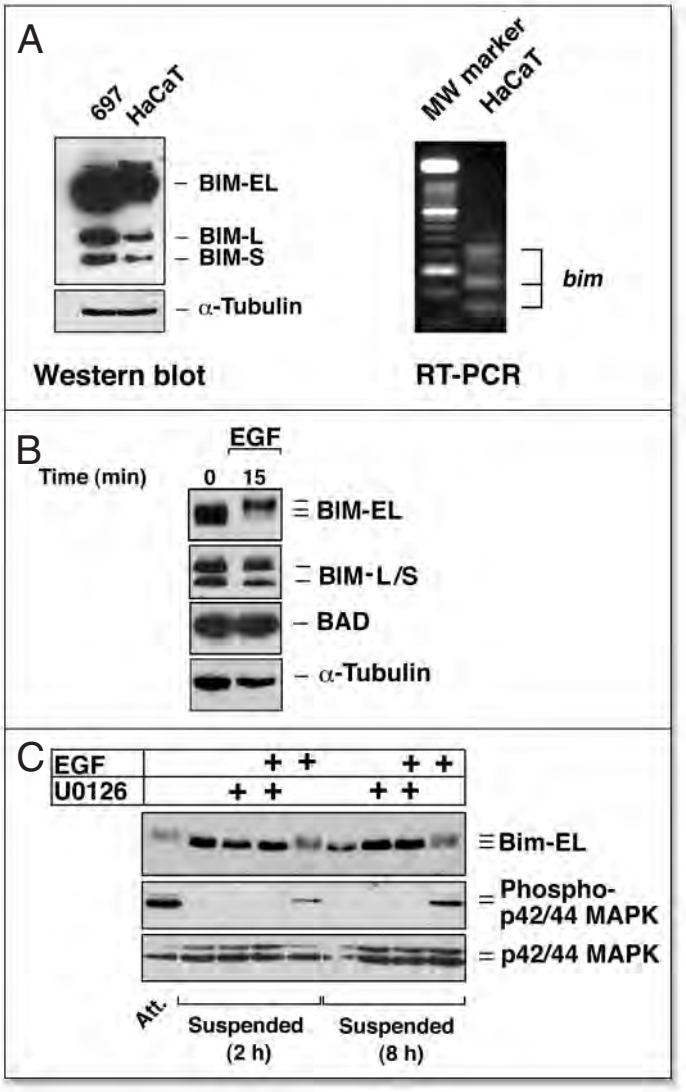

Figure 2. Expression of Bim isoforms in HaCaT cells and effects of EGF treatment on Bim expression in attached HaCaT cells (A) Expression of Bim-EL, -L and, -S in HaCaT cells as compared to pre-B 697 leukemia cells as assessed by Western blot analysis (left panel) and RT-PCR (right panel). (B) EGF-induced downregulation of Bim expression associated with gel retardation in growth factor-starved attached HaCaT cells. For control purposes, the blot was reprobed with antibodies to Bad and α-tubulin. (C) EGF-induced gel mobility shift and downregulation of Bim expression in forced suspension cultures of HaCaT cells. Cells were treated throughout the observation period (2–8 h) with EGF (10 ng/ml) and the MEK inhibitor U0126 (10 μM) as indicated. Controls are untreated attached HaCaT cells maintained in routine culture growth conditions. To assess efficacy of the MEK inhibitor U0126, its effect on MAPK phosphorylation is shown.

U0126 or the EGFR selective inhibitor AG1478. Note that the increase in cell survival through Bim downregulation in inhibitor-treated HaCaT cells represented only a fraction (17–42%) of the survival effect achieved by EGF treatment (denoted as 100% in Fig. 1B). The use of the MEK inhibitor U0126 in these experiments was based on our earlier findings that EGF treatment sustained MAPK phosphorylation in forced suspension culture, a phenomenon linked to increased HaCaT cell survival in suspension.[17,20] These experiments assigned a protective role to Bim against anoikis of epidermal keratinocytes, albeit a modest one when compared to that played by EGFR activation.

Bim expression patterns in immortalized keratinocytes during growth factor starvation and forced suspension culture. Recent studies described elevated expression of Bim in various culture conditions associated with higher sensitivity to apoptosis induction. These include not only forced suspension culture of mammary epithelial cells but also growth factor deprivation of neuronal[21] and hemopoietic cells.[10,22] These earlier results led us to examine changes in Bim expression in HaCaT cells during either growth factor deprivation or forced suspension culture. To ascertain which Bim isoforms are expressed in HaCaT cells, these experiments were initially performed in attached cells maintained in growth factor-free medium for 24 h (Fig. 2A). We observed similar patterns of Bim expression in HaCaT keratinocytes and in control 697 pre-B lymphocytes expressing high levels of Bim. Relatively strong expression of the extra long Bim isoform (Bim-EL) was contrasted by weaker signals representing the long (Bim-L) and short (Bim-S) isoforms as originally described.[9] Additional weak bands may correspond to other Bim isoforms generated by additional alternative splicing events described recently.[19] RT-PCR analysis indicated expression of at least three

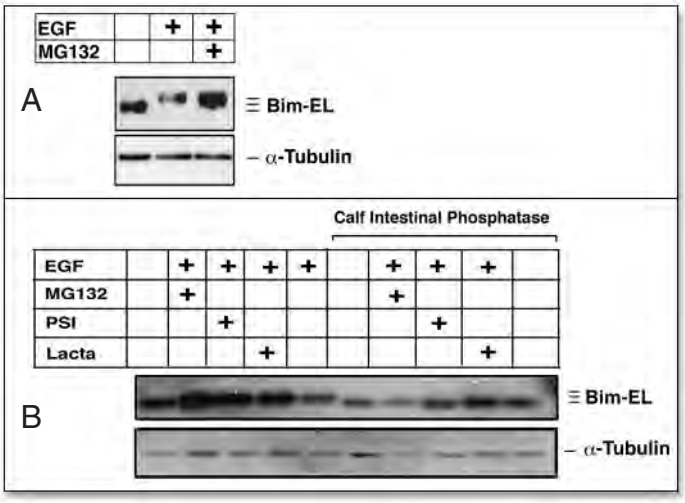

Figure 3. Effects of different proteosomal inhibitors on EGF-dependent Bim expression and gel mobility. (A) Attached growth-staved HaCaT cells were treated for 2h with MG132 (100 μM)and stimulated with EGF (10 ng/ml) for 15 min; control cells did not receive EGF. (B) Effects of phosphatase-treatment and proteasome inhibitors on Bim expression and gel migration. HaCaT cells were treated with MG 132 (100 μM), PSI (60 μM) and lactacystin (10 μM) for 2 h and then stimulated with EGF for 15 min. Aliquots of cell extracts were treated with calf intestinal phosphatase for 1h prior to immunoblot analysis using antibodies to Bim and α-tubulin as indicated. Phosphatase treatment abrogated reduced gel migration of Bim-EL caused byEGF treatment.

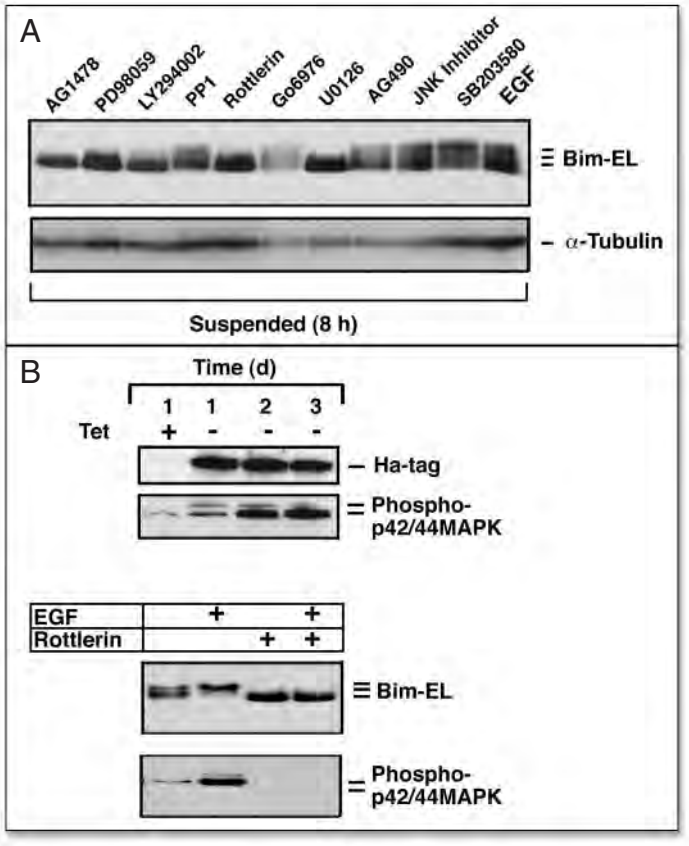

Figure 4. Effects of pharmacological inhibitors of signal transduction pathways on Bim-EL gel mobility and expression in HaCaT cells in forced suspension culture. (A) Effects of inhibitors of different signaling components. In addition to the MEK inhibitors U0126 (10 μM) and PD98059 (50 μM) the PI-3-kinase inhibitor LY294002 (10 μM) and the PKC inhibitor rottlerin (3 μM) inhibited EGF-induced effects on electrophoretic mobility of Bim. By contrast, Src inhibitor PP1 (20 μM), Jak-2 inhibitor AG490 (100 μM), JNK inhibitor (1 μM), SB203580 (10μM), and PKC α/β inhibitor Gö6976 (5 μM) had no effect. (B) Effect of rottlerin on Bim-EL expression in HaCaT cells conditionally expressing constitutively active MEK (Ha-MKK1-G1C). The upper panel demonstrates time-dependent transgene expression upon removal of tetracycline from the culture medium. The lower panel shows that rottlerin abrogated the EGF- and transgene-induced BIM-EL gel retardation and phosphorylation of MAPK at 48 h after transgene induction.

Bim transcripts encoding Bim-EL, Bim-L and, Bim-S in HaCaT keratinocytes as confirmed by diagnostic restriction enzyme analysis (Fig. 2A). In addition, we designed a separate primer set detecting BIM-EL transcripts only, which provided a robust amplification product from all RNA preparations of HaCaT cells (not shown). Next, we assessed Bim protein expression in growth factor-starved, attached HaCaT cells stimulated with EGF for 15 min (Fig. 2B). Within 15 min of EGF treatment, Bim-EL expression was reduced by 27% associated with reduced motility in SDS-PAGE gels when compared to untreated cells. By contrast, EGF treatment did not affect intensity and gel migration of the bands corresponding to Bim-L and Bim-S. Note that, in order to render the Bim-L/S bands visible (lower panel), the film was exposed considerably longer than required to detect Bim-EL expression (upper panel). Next, we assessed Bim expression in HaCaT cells placed in forced suspension cultures. As compared to attached cells maintained in routine culture conditions, i.e., media supplemented with fetal calf serum, HaCaT cells in suspension cultures performed in the absence of exogenous growth factors revealed elevated levels of Bim-EL expression (Fig. 2C). By contrast, addition of EGF attenuated Bim-EL expression at 2 h and 8 h of suspension culture accompanied by gel retardation similar to that observed in EGF-treated attached HaCaT cells. This effect was still manifest 24 h after initiation of suspension culture (not shown). Collectively, these results demonstrate that, in immortalized keratinocytes, EGFR activation was associated with reduced Bim-EL expression. These results extend similar findings reported by Reginato et al.[12] in mammary epithelial cells to epidermal keratinocytes. However, in MCF-7 cells upregulation of Bim expression in suspension cultures was ascribed primarily to enhanced steady-state Bim mRNA levels. By contrast, we observed changes in Bim-EL expression in EGF-treated HaCaT cells within minutes of growth factor exposure raising the question whether post-translational modification of Bim-EL played a more important role in EGFR-dependent effects on Bim expression in HaCaT cells.

MAPK-dependent regulation of Bim-EL expression in HaCaT keratinocytes. MAPK/ERK-dependent posttranslational Bim modification leading to reduced gel mobility of Bim-EL associated with reduced expression has been observed in phorbol ester-stimulated K562 leukemic cells.[23] In these cells, both phenomena were linked to MAPK/ERK-dependent phosphorylation of Bim on S69 followed by ubiquitin-mediated degradation. We described previously that EGFR activation is associated with sustained MAPK phosphorylation during the early stages of suspension culture.[17] Furthermore, as shown in Figure 1, HaCaT cells with reduced Bim expression are partially protected against anoikis in the presence of the U0126 MEK inhibitor. Taken together, these results led us to assess the effects of U0126 on EGF-induced Bim expression and gel migration. Consistent with the role of MEK-dependent MAPK phosphorylation, we observed that

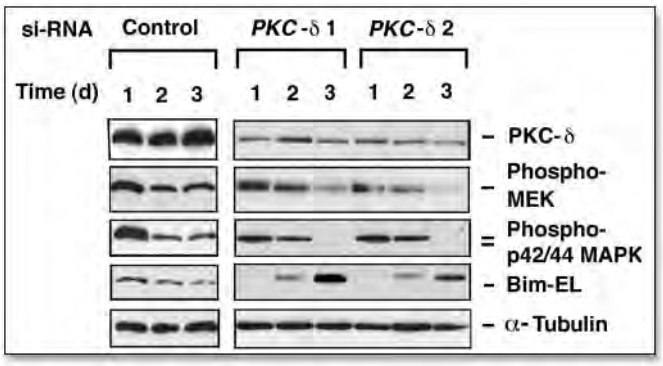

Figure 5. Effects of PKC-δ knockdown on Bim-EL expression in HaCaT cells. The effects of two different PKC-δ specific siRNA duplexes on PKC-δ (100 nM) expression in growing HaCaT keratinocytes over a period of three days after transfection. The phosphorylation of MEK, MAPK and Bim-EL expression were also determined.

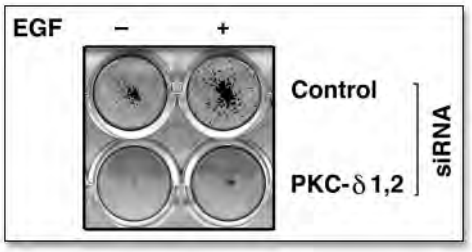

Figure 6. Decreased survival of HaCaT cells in forced suspension culture upon transfection with PKC-δ 1/2 specific siRNA duplexes. Cell survival was assessed by replating the cells on cell culture-treated plastic after 48 h of forced suspension culture either in the presence or absence of EGF (10 ng/ml) as indicated.

U0126 abrogated both, EGFR-dependent gel retardation and reduced Bim-EL protein expression, both in suspension cultures and in growth factor-starved, EGF-stimulated cells (Figs. 2C and 4A). Very similar results were achieved when using the MEK inhibitor PD98059 (Fig. 4A). To assess whether proteasomal degradation played a role in the effects of EGF on Bim-EL expression we used the proteasome inhibitor MG132. As shown in Figure 3, MG132 did not interfere with EGF-induced gel retardation of Bim-EL but markedly attenuated the inhibitory effect of EGF on Bim-EL protein expression (Fig. 3B). Collectively, these results support the notion that, in HaCaT cells, MAPK-dependent Bim-EL phosphorylation targets Bim for proteasomal degradation and, thus, leads to down-regulation of Bim-EL expression. By contrast, as assessed by RT-PCR using *Bim-EL* specific primers we did not find significant changes in *Bim-EL* transcript levels in the first 24 h of suspension culture, either in the presence or absence of EGF (not shown). In conclusion, we consider it likely that EGFR-dependent MAPK activity affects Bim protein expression in HaCaT cells primarily by post-translational modification as recently also described in mammary epithelial cells.[24]

An essential role PKC-δ in MAPK-dependent effects on Bim-EL expression. Inhibition of several other pathways previously linked to Bim phosphorylation had negligible effects on EGFR-dependent effects of Bim expression in HaCaT cells (Fig. 4A). Specifically, inhibition of JNK with a peptide inhibitor had no effect on Bim gel mobility or expression. Previous reports demonstrated Bim phosphorylation in a JNK-dependent fashion, albeit at serine/threonine residues different from those affected by MAPK,[25,26] and forced suspension culture of HaCaT cells resulted in substantial JNK phosphorylation relative to attached cells (Quadros MR, unpublished result). Similarly, inhibition of Src-kinases (PP1), JAK-kinases (AG490) and, p38 (SB203580) had no obvious effects on Bim-EL gel migration or expression in HaCaT cells. By contrast, inhibition of PI-3-kinase with LY294002 and inhibition of novel PKCs with rottlerin markedly alleviated EGF-induced Bim-EL gel retardation. Interestingly, inhibition of conventional PKCs by use of Gö6976 had no discernible effect on EGFR-dependent Bim-EL migration and expression. This result led us to investigate in more detail the role of rottlerin targets, particularly PKC-δ, in regulation of Bim-EL expression. A potential role of PI-3-kinase in this phenomenon will be investigated separately.

First, we determined whether conditional overexpression of a constitutively active MEK1 construct (MKK-G1C) would obviate the effect of rottlerin on Bim-EL expression (Fig. 4B). As shown in the upper panel of (Fig. 4B), expression of the hemagglutinin-tagged transgene was markedly induced in permissive conditions, i.e., absence of tetracycline from the culture medium. As expected, transgene expression also resulted in robust MAPK phosphorylation and partial Bim-EL gel shift even in the absence of exogenous EGF (Fig. 4B; lower panel). However, addition of rottlerin abrogated both effects, i.e., not only the Bim-EL gel shift but also MAPK phosphorylation either in the absence or presence of EGF. This result raised the issue which target of rottlerin controls MAPK phosphorylation in HaCaT cells.

As PKC-δ is the best-known rottlerin target, we decided to downregulate PKC-δ by RNA interference and determine the effects of this treatment on both, MAPK phosphorylation and Bim-EL gel migration and expression. As shown in Figure 5, progressive down-regulation of PKC-δ expression was achieved over three days after transfection of two distinct PKC-δ specific siRNA duplexes. In contrast, control cells transfected with irrelevant siRNA revealed no significant change in PKC-δ expression in this time frame. Importantly, siRNA-mediated downregulation of PKC-δ was accompanied by marked accumulation of Bim-EL in the transfected HaCaT cells. Furthermore, MAPK phosphorylation progressively decreased and was negligible 3 days after transfection of HaCaT cells with either of the two PKC-δ targeted siRNAs. Finally, MEK phosphorylation similarly decreased upon PKC-δ siRNA treatment of HaCaT keratinocytes. These results reinforce the notion that PKC-δ activity controls Bim expression in HaCaT keratinocytes, at least in part, by modulating MEK/MAPK phosphorylation. Next, we determined the effects of siRNA-mediated downregulation of PKC-δ on MEK/MAPK phosphorylation and Bim electrophoretic mobility upon short-term stimulation of attached HaCaT cells with EGF. In this setting, PKC-δ downregulation had only a moderate effect on MAPK phosphorylation and no measurable effect on Bim gel retardation and expression (results not shown). It seems possible that the partial downregulation of PKC-δ by siRNA treatment is not sufficient to block MAPK and Bim phosphorylation upon short-term EGF treatment whereas treatment with rottlerin is.

If PKC-δ activity is required for maintaining steady-state MAPK phosphorylation in HaCaT cells, abrogating PKC-δ expression should compromise HaCaT cell survival in forced suspension cultures. We chose to address this question in suspension cultures because MAPK activity is not rate-limiting to HaCaT cell survival in the attached state.[17] To maximize the siRNA effect we used a mixture of PKC-δ1 and PKC-δ2 siRNAs to transfect HaCaT cells followed by 48 h of forced suspension culture in the presence and absence of

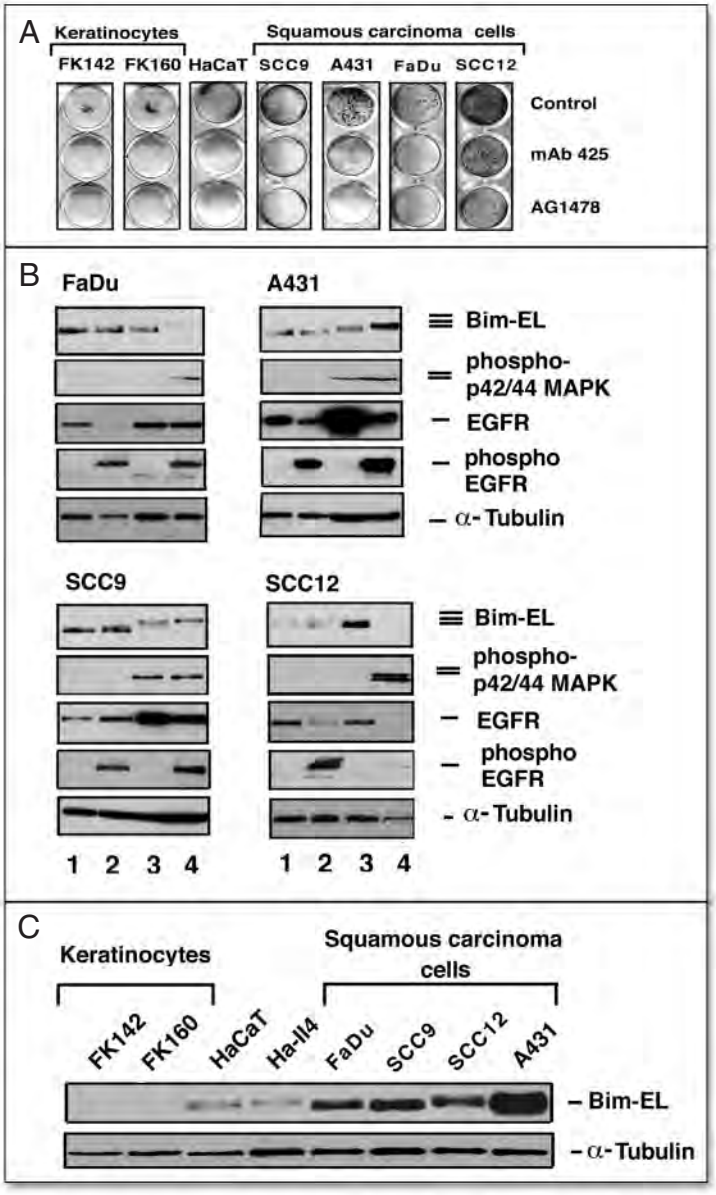

Figure 7. Anoikis sensitivity and regulation of Bim-EL expression in keratinocytes representing different stages of squamous cell carcinoma progression. (A) Effect of EGFR blockade by either EGFR antagonistic antibody 425 (10 μg/ml) or EGFR tyrosine kinase inhibitor AG1478 (10 μM) on survival of normal keratinocytes, immortalized keratinocytes (HaCaT) and SCC lines in suspension. Cells were reseeded after 48 h of suspension culture on tissue culture-treated plastic and surviving, reattached cells were stained 24 h after reseeding. (B) Modulation of Bim-EL expression and gel mobility by EGFR activation in SCC lines. Attached, growth factor-starved cells (lane 1) were stimulated with EGF (2-4) for 15 min in the presence or absence of either AG1478 (lane 2) or U0126 (lane 3). (C) Bim-EL expression in growth factor-starved, attached normal and malignant epithelial cell lines. Note absence of signal in normal keratinocyte cell extracts.

EGF. Cell survival was assayed by reseeding cells on cell culture-treated plastic and staining of reattached cells with crystal violet 24 h after reseeding. These experiments showed that siRNA-mediated downregulation of PKC-δ markedly reduced the number of cells that could be rescued following forced suspension culture when compared to mock-transfected cells (Fig. 6). Thus, PKC-δ activity not only modulates MAPK phosphorylation but also supports survival of HaCaT cells in suspension culture. This result is consistent with previous reports that PKC-δ activity supports the RAS/RAF/MEK/MAPK axis in cells as diverse as COS-1 and MCF-7.[27-29]

In keratinocytes, however, a wealth of previous evidence has implicated PKC-δ in the differentiation process[30,31] and even in apoptosis induction;[32-34] most of these previous studies were performed by characterizing the effects of deregulated PKC-δ activity on keratinocyte biology. To reconcile these apparently divergent results, we suggest that PKC-δ may serve a dual role in regulating apoptosis susceptibility of keratinocytes depending on activity levels. Whereas we observed that a moderate level of PKC-δ activity serves an anti-apoptotic role, elevated PKC-δ activity during keratinocyte differentiation may occur as a consequence of caspase activation and amplify the apoptotic process.[35,36]

Regulation of Bim-EL expression in normal keratinocytes and squamous carcinoma cells. To ascertain whether Bim-EL regulation as observed for HaCaT cells was shared in cells representing different stages of squamous cell carcinoma progression, we tested several normal primary keratinocyte cultures and squamous carcinoma cell lines. To varying degrees, all of these cell lines revealed accelerated rates of anoikis upon EGFR blockade as determined by their ability to reattach and grow after protracted culture (24–48 h) in the anchorage-independent state (Fig. 7A). As expected, the transformed cell lines exhibited a tendency towards increased survival when compared to normal keratinocytes or HaCaT cells. As in HaCaT cells, EGF treatment led to robust MAPK phosphorylation in all SCC lines accompanied by Bim-EL gel shift (Fig. 7B). In fact, we observed a strict correlation between MAPK phosphorylation and Bim gel shift in these cells consistent with an obligatory role of MAPK activation in Bim phosphorylation in this cell type. It should be noted, however, that in one of the four cell lines tested (A431), Bim-EL gel shift was not associated with reduction in expression. Interestingly, in contrast to HaCaT cells and the SCC lines tested, two normal keratinocyte strains expressed markedly less Bim, not detectable by Western blot analysis under the conditions chosen (Fig. 7C). We conclude that Bim-EL expression is negatively regulated by EGFR activation in 3 of 4 cell lines representing the full range of squamous cell carcinoma progression. For reasons unknown, however, Bim-EL expression appears to be substantially higher in malignant as compared to normal cultured cells in this lineage.

In conclusion, this study demonstrates that, as in mammary epithelial cells, survival of immortalized and transformed keratinocytes is enhanced by downregulation of Bim through EGFR activation and MEK/MAPK phosphorylation. Furthermore, our results highlight a novel role of PKC-δ in MEK/MAPK-activation and downregulation of Bim expression in HaCaT keratinocytes.

References

1. Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 1994; 124:619-26.
2. Frisch SM, Vuori K, Ruoslahti E, Chan-Hui PY. Control of adhesion-dependent cell survival by focal adhesion kinase. J Cell Biol 1996; 134:793-9.
3. Attwell S, Roskelley C, Dedhar S. The integrin-linked kinase (ILK) suppresses anoikis. Oncogene 2000; 19:3811-5.
4. Wang SC, Makino K, Xia W, Kim JS, Im SA, Peng H, Mok SC, Simgletary SE, Hung MC. DOC-2/hDab-2 inhibits ILK activity and induces anoikis in breast cancer cells through an Akt-independent pathway. Oncogene 2001; 20:6960-4.
5. Valentinis B, Morrione A, Peruzzi F, Prisco M, Reiss K, Baserga R. Anti-apoptotic signaling of the IGF-I receptor in fibroblasts following loss of matrix adhesion. Oncogene 1999; 18:1827-36.
6. Rodeck U, Jost M, DuHadaway J, Kari C, Jensen PJ, Risse B, Ewert DL. Regulation of Bcl-xL expression in human keratinocytes by cell-substratum adhesion and the epidermal growth factor receptor. Proc Natl Acad Sci USA 1997; 94:5067-72.
7. Stoll, SW, Benedict M, Mitra R, Hiniker A, Elder JT, Nunez G. EGF receptor signaling inhibits keratinocyte apoptosis: Evidence for mediation by Bcl-XL. Oncogene 1998; 16:1493-9.

8. Gilmore AP, Valentijn AJ, Wang P, Ranger AM, Bundred N, O'Hare MJ, Wakeling A, Korsmeyer SJ, Streuli CH. Activation of BAD by therapeutic inhibition of epidermal growth factor receptor and transactivation by insulin-like growth factor receptor. J Biol Chem 2002; 277:27643-50.
9. O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S, Huang DC. Bim: A novel member of the Bcl-2 family that promotes apoptosis. EMBO Journal 1998; 17:384-95.
10. Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Kontgen F, Adams JM, Strasser A. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science 1999; 286:1735-8.
11. Bouillet P, Cory S, Zhang LC, Strasser A, Adams JM. Degenerative disorders caused by Bcl-2 deficiency prevented by loss of its BH3-only antagonist Bim. Dev Cell 2001; 1:645-53.
12. Reginato MJ, Mills KR, Paulus JK, Lynch DK, Sgroi DC, Debnath J, Muthuswamy SK, Brugge JS. Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. Nat Cell Biol 2003; 5:733-40.
13. Murthy U, Basu A, Rodeck U, Herlyn M, Ross AH, Das M. Binding of an antagonistic monoclonal antibody to an intact and fragmented EGF-receptor polypeptide. Arch Biochem Biophys 1987; 252:549-60.
14. Rodeck U, Herlyn M, Herlyn D, Molthoff C, Atkinson B, Varello M, Steplewski Z, Koprowski H. Tumor growth modulation by a monoclonal antibody to the epidermal growth factor receptor: Immunologically mediated and effector cell-independent effects. Cancer Res 1987; 47:3692-6.
15. McNeill H, Jensen PJ. A high-affinity receptor for urokinase plasminogen activator on human keratinocytes: Characterization and potential modulation during migration. Cell Regul 1990; 1:843-52.
16. Quadros MR, Peruzzi F, Kari C, Rodeck U. Complex regulation of signal transducers and activators of transcription 3 activation in normal and malignant keratinocytes. Can Res 2004; 64:3934-9.
17. Jost M, Huggett TM, Kari C, Rodeck U. Matrix-independent survival of human keratinocytes through an EGF receptor/MAPK-Kinase-dependent pathway. Mol Biol Cell 2001; 12:1519-27.
18. Rodeck U, Bossler A, Kari C, Humphreys CW, Gyorfi T, Maurer J, Thiel E, Mensen HD. Expression of the *wt1* Wilms' tumor gene by normal and malignant human melanocytes. Int J Cancer 1994; 59:78-82.
19. Marani M, Tenev T, Hancock D, Downward J, Lemoine NR. Identification of novel isoforms of the BH3 domain protein Bim which directly activate Bax to trigger apoptosis. Mol Cell Biol 2002; 22:3577-89.
20. Jost M, Huggett TM, Kari C, Boise LH, Rodeck U. Epidermal growth factor receptor-dependent control of keratinocyte survival and Bcl-xL expression through a MEK-dependent pathway. J Biol Chem 2001; 276:6320-6.
21. Whitfield J, Neame SJ, Paquet L, Bernard O, Ham J. Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. Neuron 2001; 29:629-43.
22. Villunger A, Scott C, Bouillet P, Strasser A. Essential role for the BH3-only protein Bim but redundant roles for Bax, Bcl-2, and Bcl-w in the control of granulocyte survival. Blood 2003; 101:2393-400.
23. Luciano F, Jacquel A, Colosetti P, Herrant M, Cagnol S, Pages G, Auberger P. Phosphorylation of Bim-EL by Erk1/2 on serine 69 promotes its degradation via the proteasome pathway and regulates its proapoptotic function. Oncogene 2003; 22:6785-93.
24. Wang P, Gilmore AP, Streuli CH. Bim is an apoptosis sensor that responds to loss of survival signals delivered by epidermal growth factor but not those provided by integrins. J BiolChem 2004; 279:41280-5.
25. Lei K, Davis RJ. JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. Proc Natl Acad Sci USA 2003; 100:2432-7.
26. Putcha GV, Le S, Frank S, Besirli CG, Clark K, Chu B, Alix S, Youle RJ, LaMarche A, Maroney AC, Johnson EM. JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis. Neuron 2003; 38:899-914.
27. Ueda Y, Hirai S, Osada S, Suzuki A, Mizuno K, Ohno S. Protein kinase C activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. J Biol Chem 1996; 271:23512-9.
28. Schonwasser DC, Marais RM, Marshall CJ, Parker PJ. Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. Mol Cell Biol 1998; 18:790-8.
29. Keshamouni VG, Mattingly RR, Reddy KB. Mechanism of 17-beta-estradiol-induced Erk1/2 activation in breast cancer cells. A role for HER2 AND PKC-delta. J Biol Chem 2002; 277:22558-65.
30. Efimova T, LaCelle P, Welter JF, Eckert RL. Regulation of human involucrin promoter activity by a protein kinase C, Ras, MEKK1, MEK3, p38/RK, AP1 signal transduction pathway. J Biol Chem 1998; 273:24387-95.
31. Efimova T, Deucher A, Kuroki T, Ohba M, Eckert RL. Novel protein kinase C isoforms regulate human keratinocyte differentiation by activating a p38 delta mitogen-activated protein kinase cascade that targets CCAAT/enhancer-binding protein alpha. J Biol Chem 2002; 277:31753-60.
32. Li L, Lorenzo PS, Bogi K, Blumberg PM, Yuspa SH. Protein kinase Cdelta targets mitochondria, alters mitochondrial membrane potential, and induces apoptosis in normal and neoplastic keratinocytes when overexpressed by an adenoviral vector. Mol Cell Biol 1999; 19:8547-58.
33. Denning MF, Wang Y, Tibudan S, Alkan S, Nickoloff BJ, Qin JZ. Caspase activation and disruption of mitochondrial membrane potential during UV radiation-induced apoptosis of human keratinocytes requires activation of protein kinase C. Cell Death and Diff 2002; 9:40-52.
34. Papp H, Czifra G, Bodo E, Lazar J, Kovacz I, Alekza M, Juhazs I, Acs P, Sipka S, Kovacs L, Blumberg PM, Biro T. Opposite roles of protein kinase C isoforms in proliferation, differentiation, apoptosis, and tumorigenicity of human HaCaT keratinocytes. Cell Mol Life Sci 2004; 61:1095-105.
35. Denning MF, Wang Y, Nickoloff BJ, Wrone-Smith T. Protein kinase Cdelta is activated by caspase-dependent proteolysis during ultraviolet radiation-induced apoptosis of human keratinocytes. J Biol Chem 1998; 273:29995-30002.
36. Okuyama R, Nguyen BC, Talora C, Ogawa E, de Vignano AT, Lioumi M, Chiorino G, Tagami H, Woo M, Dotto GM. High commitment of embryonic keratinocytes to terminal differentiation through a Notch1-caspase 3 regulatory mechanism. Dev Cell 2004; 6:551-62.

JCS ePress online publication date 3 January 2006

E-cadherin loss promotes the initiation of squamous cell carcinoma invasion through modulation of integrin-mediated adhesion

Weitian Zhang[1,*], Addy Alt-Holland[1,*], Alexander Margulis[1], Yulia Shamis[1], Norbert E. Fusenig[2], Ulrich Rodeck[3] and Jonathan A. Garlick[1,‡]

[1]Division of Cancer Biology and Tissue Engineering, Department of Oral and Maxillofacial Pathology, School of Dental Medicine, Tufts University, 55 Kneeland Street, Boston, MA 02111, USA
[2]German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany
[3]Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, 233 South 10th Street, Philadelphia, PA 19107, USA
*These authors contributed equally to this work
‡Author for correspondence (e-mail: jonathan.garlick@tufts.edu)

Accepted 5 October 2005
Journal of Cell Science 119, 283-291 Published by The Company of Biologists 2006
doi:10.1242/jcs.02738

Summary

Much remains to be learned about how cell-cell and cell-matrix interactions are coordinated to influence the earliest development of neoplasia. We used novel 3D human tissue reconstructs that mimic premalignant disease in normal epidermis, to directly investigate how loss of E-cadherin function directs conversion to malignant disease. We used a genetically tagged variant of Ha-Ras-transformed human keratinocytes (II-4) expressing dominant-interfering E-cadherin fusion protein (H-2k^d-Ecad). These cells were admixed with normal human keratinocytes and tumor cell fate was monitored in 3D reconstructed epidermis upon transplantation to immunodeficient mice. Tumor initiation was suppressed in tissues harboring control- and mock-infected II-4 cells that lost contact with the stromal interface. By contrast, H-2k^d-Ecad-expressing cells persisted at this interface, thus enabling incipient tumor cell invasion upon in vivo transplantation. Loss of intercellular adhesion was linked to elevated cell surface expression of α2, α3 and β1 integrins and increased adhesion to laminin-1 and Types I and IV collagen that was blocked with β1-integrin antibodies, suggesting that invasion was linked to initial II-4 cell attachment at the stromal interface. Collectively, these results outline a novel aspect to loss of E-cadherin function that is linked to the mutually interdependent regulation of cell-cell and cell-matrix adhesion and has significant consequences for the conversion of premalignancy to cancer.

Key words: E-cadherin, Integrin, Squamous cell carcinoma, 3D cultures, Premalignant

Introduction

Cell fate decisions in whole organisms are largely controlled by signals imparted by the tissue microenvironment. This occurs through a complex network of molecular signals that is directed either by interactions between neighboring cells or with extracellular matrix components or by stimulation of cell surface receptors by soluble factors. This multitude of environmental signals provides an efficient system to control cell behavior and maintain tissue homeostasis. Conversely, cancer development may be considered as a breakdown of microenvironmental control of cell behavior (Bissell and Radisky, 2001). Consistent with this notion, both integrin-mediated cell-matrix adhesion and cadherin-mediated cell-cell adhesion are frequently altered in tumor cells as a result of loss or gain of specific adhesion receptors. For example, the cell-cell adhesion receptor E-cadherin is frequently lost during advanced stages of epithelial cancer progression (Perl et al., 1998; Birchmeier and Behrens, 1994) and this is considered to be an important step in invasion and metastasis of epithelial tumor cells (Bissell and Radisky, 2001; Cavallaro and Cristofori, 2004; Cano et al., 2000). However, it is not well understood how perturbation of cell adhesion networks can influence the initial stages of cancer progression as premalignant-to-malignant conversion occurs.

A complex epithelium such as the epidermis provides a model system that is well suited to study the networks of structural and contextual signals that govern the orderly execution of normal proliferation and differentiation programs (Jamora and Fuchs, 2002). During the earliest, intraepithelial stages of squamous cell carcinoma development, small nests of aberrant, dysplastic tumor cells are exposed to and affected by the same positional signals that govern normal epithelial cell behavior (Dlugosz et al., 2002). To better understand this interplay we have developed three-dimensional (3D) human tissue models that allow us to monitor premalignant, intraepithelial (IE) stages of squamous carcinoma progression in the context of an in-vivo-like stratified epithelium (Alt-Holland et al., 2005). We described previously that interactions between IE tumor cells and neighboring normal cells can lead to suppression of early neoplastic progression by inducing a state of 'intraepithelial dormancy' (Javaherian et al., 1998). This dormant state could be overcome by altered tissue dynamics in response to the tumor promoter TPA (Karen et al., 1999), ultraviolet (UV) irradiation (Mudgil et al., 2003), decreased adhesive interactions between tumor cells and adjacent epithelia

(Vaccariello et al., 1999) or by enabling tumor cells to interact with basement membrane (BM) proteins (Andriani et al., 2004). These observations implied that alterations in the ability of IE tumor cells to adhere to adjacent cells or in their capacity to persist at the stromal interface were of crucial importance to their invasive potential. Concurrently, other studies performed in conventional two-dimensional (2D) cultures, provided evidence for mutual interdependence between cadherin- and integrin-mediated adhesion events in cancer cells (Avizienyte et al., 2002; Zhang et al., 2003; Yano et al., 2004). However, the functional consequences of such crosstalk between these distinct adhesive pathways during the earliest stages of cancer development in 3D tissues or in vivo remain largely unknown.

The present study provides new insights into the dynamic equilibrium between cell-cell and cell-matrix adhesive events in early epithelial tumor progression. Specifically, ablation of E-cadherin function in cells with malignant potential imparted an invasive phenotype to these cells by allowing them to overcome microenvironmental constraints on their progression. This phenomenon was associated with retention of E-cadherin-deficient cells at the epithelial-stromal interface in epidermal reconstructs. Furthermore, increased retention at this interface co-segregated with enhanced adhesion of E-cadherin-deficient cells to extracellular matrix components including laminin, fibronectin and Type IV collagen in vitro. Finally, we describe increased expression of integrin subunits likely to mediate adhesion in E-cadherin-deficient cells to collagen Types I and IV at this interface. Collectively, our results point to a novel aspect of E-cadherin loss in epithelial tumor progression that is not only related to severing cell-cell adhesion but also is associated with increased cell-matrix adhesion of these cells. The functional consequence of enhanced cell-matrix adhesion is the initial attachment and retention of these cells at the epithelial-stromal interface, thus providing the appropriate microenvironmental conditions for incipient tumor cell invasion.

Results

Loss of E-cadherin enables premalignant-to-malignant conversion upon incipient tumor cell invasion

Our previous studies showed that II-4 cells were non-tumorigenic when placed in epidermal reconstructs with human epidermal keratinocytes (HEK) because of their microenvironmental growth suppression and were shed from the epithelial surface (Javaherian et al., 1998). Based on this earlier observation, we first determined if loss of E-cadherin function could circumvent this constraint by altering the dynamics of II-4 cell behavior when grown in the context of normal, differentiating epidermal cells in 3D reconstructs. Epidermal reconstructs were established in vitro with either control II-4 cells expressing pBabe or H-2k^{d}-EcadC25 or with II-4 cells expressing a dominant-interfering E-cadherin (H-2k^{d}-Ecad). The H-2k^{d}-Ecad transgene has previously been described to interfere with E-cadherin function in normal keratinocytes (Zhu and Watt, 1996) and II-4 cells (Margulis et al. 2005a). This occurs as overexpression of this fusion protein leads to destabilization of endogenous E-cadherin-β-catenin complexes upon the cytoplasmic sequestration of β-catenin by the dominant-interfering transgene and to loss of E-cadherin-mediated intercellular adhesion. By contrast, the H-2k^{d}-EcadC25 used as a control, lacks the β-catenin binding region in the intracellular domain of E-cadherin and has no discernible effect on E-cadherin function. To model a specific IE tumor cell load, HEK were mixed at a 4:1 ratio with II-4 cell variants expressing either of these vectors in 3D tissue constructs and were transplanted as surface grafts to the dorsum of nude mice for 4 weeks. Upon transplantation, only grafts containing HEK/H-2k^{d}-Ecad II-4 mixtures generated clinically apparent lesions (Fig. 1C) whereas reconstructs harboring pBabe and H-2k^{d}-Ecad-C25 gave rise to normal human epidermis (Fig. 1A,B respectively). HEK/H-2k^{d}-Ecad-II-4 mixtures gave rise to raised, nodular lesions with focal areas of erythema (Fig. 1C, arrow). H-2k^{d}-Ecad-II-4 tumor

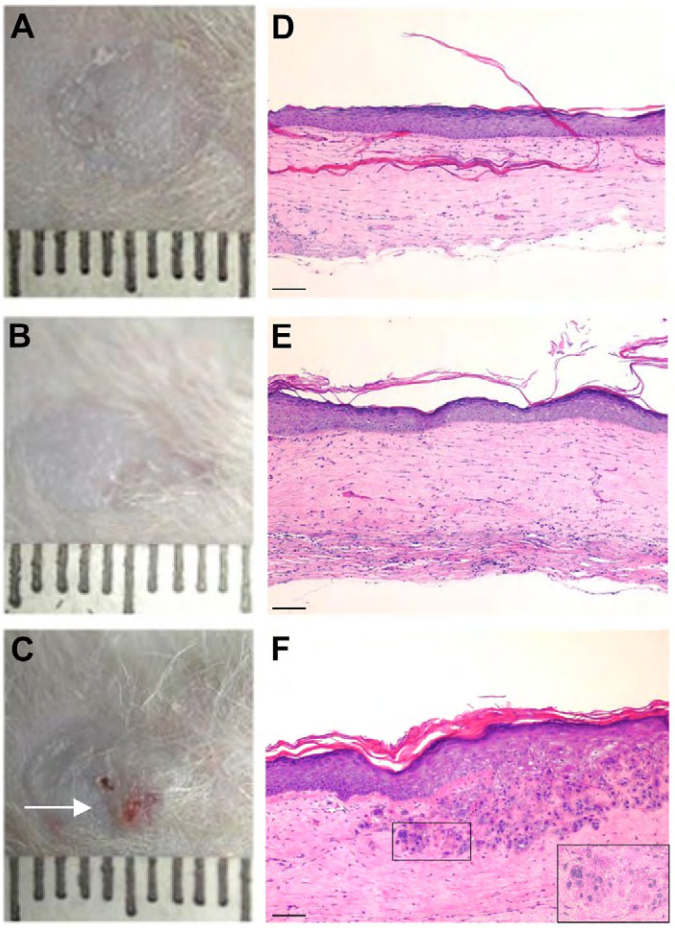

Fig. 1. Invasive tumor formation after in vivo transplantation was dependent upon loss of E-cadherin. (A-C) Clinical appearance of 3D surface transplants (left panel): 3D tissues were engineered in vitro as 4:1 mixtures of HEK and either pBabe-, H-2k^{d}-EcadC25- or H-2k^{d}-Ecad-II-4-expressing cells and transplanted to the dorsum of nude mice for 4 weeks. Grafts harboring E-cadherin-deficient, H-2k^{d}-Ecad-expressing II-4 cells generated nodular tumors with focal areas of erythema (C, arrow). By contrast, grafts containing II-4-pBabe (A) or H-2k^{d}-EcadC25-expressing cells (B) generated normal grafts without any evidence of tumor formation. (D-F) Histological appearance of 3D surface transplants. Tumors generated in transplants of 4:1 mixtures of HEK and H2k^{d}-Ecad-expressing II-4 cells demonstrated invasion of individual cells (F, inset) and small clusters of poorly differentiated tumor cells (F). By contrast, grafted mixtures of HEK with either pBabe- (D) or H-2k^{d}-EcadC25-expressing II-4 cells (E) generated normal epithelia without any evidence of residual tumor cells. Bars, 20 μm.

cells invaded into the dermis as small clusters or as individual cells (Fig. 1F, inset). By contrast, mixtures of control HEK/pBabe-II-4 (Fig. 1A,D) or HEK/H-2k^{d}-EcadC25-II4 (Fig. 1B,E) generated normal epidermal grafts with intact epithelial architecture in which no tumor cells were visible. The fate and phenotype of H-2k^{d}-Ecad-expressing II-4 cells was further studied by double immunostaining for the β-gal marker gene and β-catenin to determine that transgene expression was maintained in vivo and led to loss of adherens junctions from cell-cell borders (Fig. 2). β-gal-positive II-4 cells were absent from tissues that originally harbored mixtures of HEK with either the control pBabe- (Fig. 2A) or H-2k^{d}-EcadC25-expressing II-4 cells (Fig. 2B). In these transplants, β-catenin was detected only at cell-cell borders of HEK (Fig. 2A,B). By contrast, large groups of β-gal-positive, II-4 cells were apparent beneath the dermal/epidermal junction in HEK/H-2k^{d}-Ecad-II-4 grafts (Fig. 2C) indicating that these cells had traversed the BM and had invaded into the dermal compartment. Furthermore, these grafts showed cytoplasmic localization of β-gal (Fig. 2C) and β-catenin (Fig. 2D) in tumor cells indicating that loss of E-cadherin function was associated with redistribution of β-catenin from cell junctions in invading cells. Moreover, the majority of large groups of invading β-gal-positive tumor cells localized beneath the dermal/epidermal junction presented increased expression of the α2 integrin subunit (Fig. 2E). By contrast, in transplants that originally harbored the mixture of HEK with either the control pBabe (Fig. 2F) or H-2k^{d}-EcadC25-expressing II-4 cells (not shown), α2 integrin expression was restricted only to the basal HEK that were layered on the epithelial-stromal interface. This suggested that altered integrin-mediated adhesion may play a role in the invasive properties of these tumor cells. In summary, these results underscore that invasion of II-4 cells was contingent upon loss of E-cadherin function and required the capacity of these cells to persist in the tissue despite loss of adhesion with adjacent cells. This result raised the question as to how H-2k^{d}-Ecad-II-4 cells were retained at the BM interface as a precondition for tumor cell invasion.

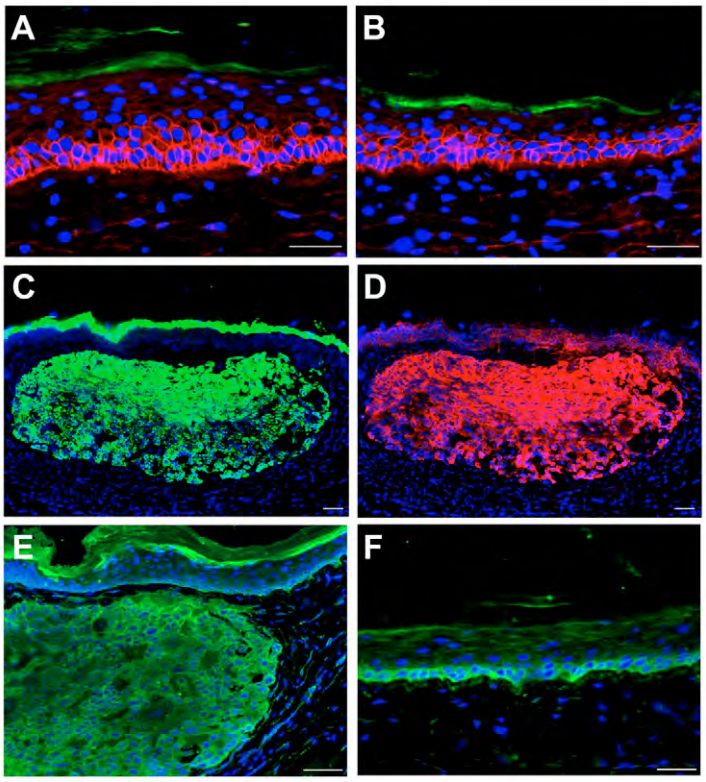

Fig. 2. Tumor cell invasion was linked to the cytoplasmic redistribution of β-catenin and increased expression of α2 integrin subunit. (A-F) Four weeks after in vivo transplantation of 4:1 mixtures, excised tissues were stained by double immunofluorescence for β-gal (green) and β-catenin (red). In transplanted mixtures comprised of HEK and control pBabe- (A) or EcadC25-expressing II-4 cells (B), β-gal-positive II-4 cells were not present and β-catenin was localized at cell-cell borders of the HEK cells. By contrast, as can be seen by the same immunostaining pattern, mixtures of HEK and H2k^{d}-Ecad-expressing II-4 cells demonstrated islands of invasive tumor cells that showed cytoplasmic co-localization of β-gal (C, green) and β-catenin (D, red), indicating that II-4 cell invasion was associated with loss of β-catenin from cell junctions and abrogation of cell-cell adhesion. The invading clusters of tumor cells showed increased expression of α2 integrin subunit (E, green), whereas in control grafts comprised of HEK and control pBabe-II-4 cells, α2 integrin expression was limited to the basal HEK at the epithelial-stromal interface (F, green). Bars, 10 μm.

Loss of E-cadherin function enhances attachment of II-4 cells to the epithelial-stromal interface and is a prerequisite for tumor cell invasion

To address this issue, we assessed the distribution of II-4 cell variants in cell mixtures in 3D in vitro constructs just prior to transplantation. Mixtures of HEK with any of the three types of II-4 cells were cultured at 4:1 ratios at an air-liquid interface for 7 days. The distribution of tumor cells in reconstructs was then analyzed by double immunofluorescent staining for β-gal and Type IV collagen to identify the II-4 cells and the BM interface, respectively. Reconstructs harboring H-2k^{d}-Ecad-expressing cells demonstrated individual β-gal-positive, II-4 cells in the basal layer of the epithelium, adjacent to Type IV collagen seen at the epithelial-stromal interface (Fig. 3C, arrow). By contrast, no β-gal-positive cells were seen at this interface in tissues containing the HEK/pBabe-II-4 (Fig. 3A) or HEK/H-2k^{d}EcadC25-II-4 mixtures (Fig. 3B). Consistent with their eventual loss by shedding from the epithelial surface, these control cells were exclusively observed in the suprabasal cell layers of the epithelium and had lost contact with the stromal interface (Fig. 3A,B, arrows). These findings suggested that loss of E-cadherin altered the adhesive repertoire of these cells, thus creating permissive conditions for their attachment and persistence in the basal layer. Moreover, these results identify two separate but mutually interdependent mechanisms by which loss of E-cadherin function may support initiation of squamous cell carcinoma progression. First, absence of E-cadherin function allowed these cells to evade tumor suppression mediated by cell-cell contact that could have prevented their persistence after in vivo transplantation. Secondly, loss of E-cadherin function enabled small numbers of II-4 cells to adhere to the stromal interface in vitro and, thus, to manifest their invasive potential after in vivo transplantation.

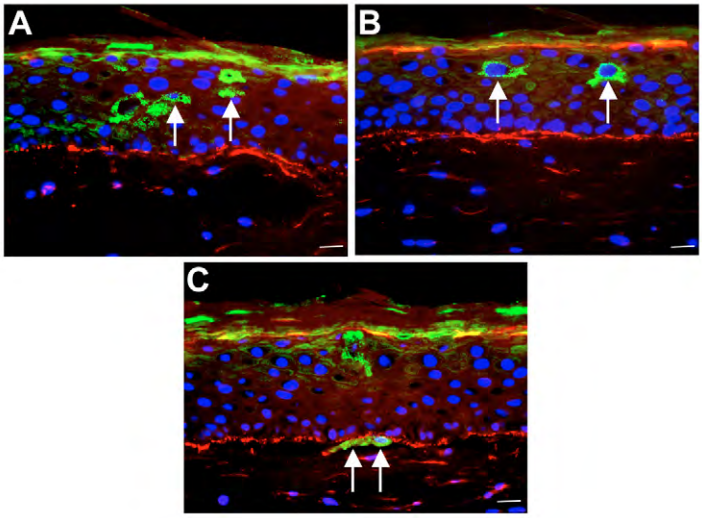

Fig. 3. Invasion of cell-cell adhesion-deficient II-4 cells was preceded by their attachment and localization at the epithelial-stromal interface in 3D in vitro tissues. In vitro 3D cultures were constructed as 4:1 mixtures of HEK with either pBabe-, H-2k^{d}-EcadC25- or H-2k^{d}-Ecad-II-4 cells and were grown at an air-liquid interface for 7 days. Tissues were stained by double immunofluorescence for the basement membrane component Type IV collagen (red) and β-gal (green). Constructs harboring mixtures of HEK with either pBabe- (A, arrows) or H-2k^{d}-EcadC25-expressing II-4 cells (B, arrows) demonstrated tumor cells that were limited to the suprabasal layers of the epithelium. By contrast, tissues harboring H-2k^{d}-Ecad-II-4 cells demonstrated β-gal-positive cells in the basal layer of the epithelium, adjacent to the Type IV collagen present at the epithelial-stromal interface (C, arrows). Bars, 10 μm.

Loss of E-cadherin function is associated with enhanced cell-matrix adhesion

To determine whether the attachment of E-cadherin-deficient cells to the stromal interface prior to tumor cell invasion was due to changes in their adhesion to extracellular matrix components, short-term attachment assays were performed in monolayer cultures. H-2k^{d}-Ecad-expressing II-4 cells demonstrated a significant increase in adhesion to surfaces coated with ECM substrates when compared with control pBabe- and H-2k^{d}EcadC25-expressing II-4 cells (Fig. 4). This effect was most pronounced when H-2k^{d}-Ecad-expressing cells were seeded on the BM constituents Type IV collagen (3.5-fold increase; Fig. 4B) and laminin (twofold increase; Fig. 4C). In addition, H-2k^{d}-Ecad-expressing II-4 cells demonstrated elevated adhesion to Type I collagen-coated (Fig. 4A) and fibronectin-coated (Fig. 4D) plates when compared with the control II-4 cells. This demonstrates that loss of E-cadherin-mediated adhesion was associated with a general increase in the repertoire of matrix-adhesive receptors on the surface of these cells.

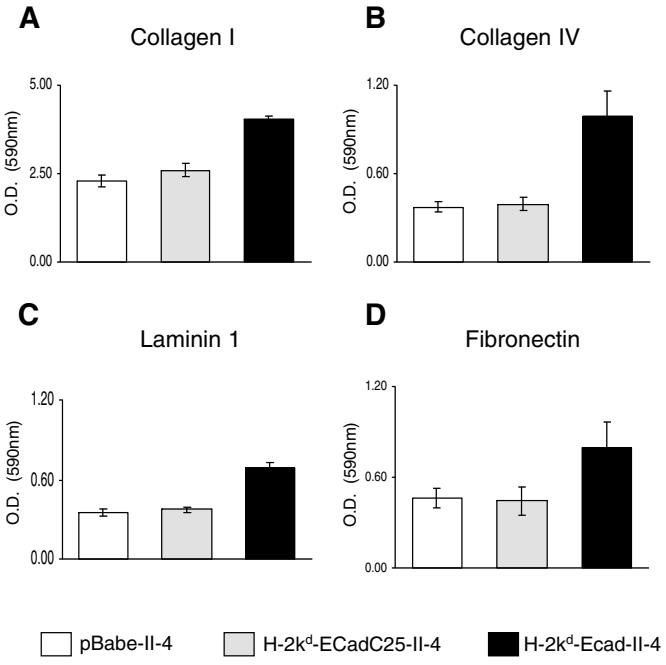

Fig. 4. Loss of E-cadherin increases II-4 cell attachment to extracellular matrix proteins. 2D cultures of pBabe-, H-2k^{d}-EcadC25- or H-2k^{d}-Ecad-II-4 cells were trypsinized briefly and replated onto plates coated with either Type I collagen (A), Type IV collagen (B), laminin-1 (C) or fibronectin (D) for 20 minutes. Attached cells were quantified by optical density determined at 590 nm. Results are calculated as the mean ± s.d. of four replicates and experiments were repeated four times. P<0.01 for H-2k^{d}-Ecad-II-4 cells vs control cells in adherence to Types I and IV collagen substrates. P<0.05 for H-2k^{d}-Ecad-II-4 cells vs control cells in adherence to laminin-1 and fibronectin substrates.

Loss of E-cadherin function in II-4 cells is associated with enhanced expression of α2, α3 and β1 integrin subunits

To address the mechanism underlying augmented matrix adhesion of H-2k^{d}-Ecad-II-4 cells, we compared the levels of specific integrin subunits expressed on the surface of the three II-4 cell lines in 2D monolayer cultures. Immunoblotting of membrane extracts revealed a marked increase in the level of α3 integrin expression in E-cadherin-deficient, H-2k^{d}-Ecad-II-4 cells, when compared with the relatively low α3 expression level present in the control pBabe-II-4 and in the H-2k^{d}EcadC25-expressing II-4 cultures (Fig. 5A). As in invading tumor cells in nude mice, a similar increase in expression was observed for the α2 integrin subunit, in H-2k^{d}-Ecad-expressing cells, when compared with control pBabe-II-4 cells and with H-2k^{d}EcadC25-expressing II-4 cells (Fig. 5A). However, in both control cultures, the expression level of α2 subunit was higher than the expression level of the α3 integrin subunit. As expected, the increased expression level of both α3 and α2 subunits was accompanied by an increased expression of the complementary β1 integrin subunit in the H-2k^{d}-Ecad-expressing cells, in comparison to β1 levels present in both control cultures.

These findings were supported by flow cytometric analysis of cell surface integrin expression of the three II-4 cell lines under investigation. H-2k^{d}-Ecad-II-4 and the control immunolabeled

pBabe-II-4 cells, and H-2k^{d}EcadC25-expressing II-4 cells were labeled with anti-α2, α3 or β1 integrin antibodies. The levels of cell surface integrin subunits α2, α3 and β1 were considerably increased in the E-cadherin-deficient, H-2k^d-Ecad-II-4 cells, when compared with levels of these cell surface integrin subunits in the control pBabe-II-4 cells (Fig. 5B, right panel). By contrast, no significant differences in cell surface integrin levels of α2, α3 and β1 subunits were identified between control pBabe-II-4 cells and H-2k^{d}EcadC25-II-4 cells (Fig. 5B, left panel).

Finally, to determine the functional significance of the increased expression of the integrin subunits in H-2k^d-Ecad-II-4 cells, short-term adherence assays to Type I and IV collagen and to laminin 1 substrates were performed in the presence or absence of β1 integrin blocking antibodies. As seen in Fig. 5C, the presence of specific β1-blocking antibodies dramatically reduced the ability of H-2k^d-Ecad-II-4 cells to adhere to Type I and IV collagens (80% and 55% decrease, respectively). By contrast, cells adhered to a lesser extent to laminin 1 and were mildly affected by the presence of β1 blocking antibodies (Fig. 5C, 20% decrease). Taken together these results demonstrate that loss of E-cadherin-mediated cell-cell adhesion was associated with increased expression of functioning cell surface α2, α3 and β1 integrin subunits. Furthermore, this increase in integrin expression is correlated with the ability of these cells to adhere rapidly to Type I and IV collagens, that are present at the epithelial-stromal interface.

Discussion

Microenvironmental cues inherent in normal tissue architecture are known to limit the malignant potential of precancerous tumor cells through an intrinsic, tissue-based control of early cancer progression (Bissell and Radisky, 2001; Alt-Holland et al., 2005). Consistent with this notion, we have previously established that interactions between tumor cells and adjacent normal keratinocytes induce a state of 'intraepithelial dormancy' in which premalignant tumor cells embedded in the stratifying epidermis withdraw from the cell cycle, lose attachment to the basement membrane and are eventually lost along with terminally differentiating keratinocytes (Javaherian et al., 1998). Here we report that this barrier to early squamous carcinoma progression can be overcome through previously unknown molecular events incurred upon loss of E-cadherin function in epithelial cells with malignant potential. Specifically, disruption of E-cadherin function in H-Ras-transformed human cell line was associated with an increase in integrin-mediated adhesion that enabled intraepithelial (IE) tumor cells to adhere to the epithelial-stromal interface in 3D tissue reconstructs. This increased adhesion set the stage for invasion of tumor cells that was observed after transplantation of skin reconstructs to immunodeficient mice. Collectively, these results suggest a novel role for E-cadherin loss in the initiation of epithelial tumor progression that arose from linkage between loss of cell-cell adhesion and gain of cell-matrix adhesion.

Both integrin-mediated cell-matrix adhesion and cadherin-mediated cell-cell adhesion are known to play central roles in tumor cell adhesion, migration, invasion, and metastasis (Cavallaro and Christofori, 2004; Friedl and Wolf, 2003). Recent studies using monolayer cultures of epithelial cancer cells revealed a complex network of molecular pathways linking cell-cell and cell-matrix adhesion. For example, integrin-mediated loss of E-cadherin from cell-cell junctions is associated with overexpression of *src* in colon carcinoma cells. In addition, integrin-linked kinase has been shown to control E-cadherin expression (Wu et al., 1999) and elements of the integrin signaling complex regulate N-cadherin-mediated adhesion (Gotzman et al., 2004). Furthermore, abrogation of E-cadherin function in breast cancer cells resulted in increased activity of the αv integrin subunit and increased cell migration (van Schliepp et al., 2000). We extend these earlier observations into 3D human tissue constructs by describing increased surface localization and function of β1 integrins upon loss of E-cadherin in II-4 cells. Upregulation of α2, α3 and β1 integrin subunits was paralleled by increased adhesion to extracellular matrix components, particularly laminin and Type IV collagen. Furthermore, only IE tumor cells that had lost functional adherens junctions, as seen by the cytoplasmic redistribution of β-catenin, were retained at the stromal interface in vitro. Thus, the present investigation demonstrates for the first time, that the mutually interdependent regulation of cell-cell and cell-matrix adhesion has significant consequences for the initial stages of epithelial cancer progression in 3D human tissues that mimic their in vivo counterparts.

The ability of E-cadherin-deficient II-4 cells to initiate invasion in the context of NHK is due specifically to increased integrin-mediated attachment to Types I and IV collagen at the epithelial-stromal interface. We have performed function-blocking studies using antibody directed against β1 integrin to show that this adhesion can be significantly limited when these cells are exposed to this blocking antibodies. Thus, increased expression and function of α2, α3 and β1 integrin subunits seen upon loss of E-cadherin function, enables adhesion of E-cadherin-deficient cells that is specific to these ECM proteins. These findings now enable us to hypothesize how the link between loss of E-cadherin and gain of specific integrin subunits allow intraepithelial tumor cells to manifest their invasive behavior. First, loss of E-cadherin and increased integrin function enables adhesion specifically to Type I collagen, which was the predominant protein present at the stromal interface when 3D cultures were seeded onto Type I collagen gels. Type IV collagen may also play a role in the retention of E-cadherin-deficient cells, as it is subsequently deposited by II-4 cells in the basal layer several days after cells are seeded into 3D tissues (Andriani et al., 2004). Interestingly, integrin-mediated binding to laminin-1 does not appear to play a role during these events, as integrin-blocking antibodies do not significantly decrease the adhesion of E-cad-deficient cells. It is possible that this laminin may play a role in adhesion through non-integrin receptors (Kim et al., 1999). Thus, only after attachment of E-cadherin-deficient cells to Type I and IV collagen at the stromal interface will these cells realize their malignant potential by overcoming local, microenvironmental constraints. This points to a crucial role for the loss of E-cadherin and acquisition of specific ECM attachment in the initiation of tumor cell invasion.

Acquisition of an invasive phenotype is a crucial event in early tumor progression, as this property is a prerequisite for connective tissue infiltration and metastatic dissemination associated with poor clinical outcomes. A molecular mechanism recognized to guard against invasion and metastasis is apoptosis or anoikis that is incurred upon loss of integrin-mediated ECM adhesion (Meredith et al., 1993; Frisch

and Francis, 1994). Anoikis has been described for both immortalized human keratinocytes with low malignant potential (Jost et al., 2001) and for SCC cell lines at a more advanced stage of tumor progression (Kim et al., 1999; Janes and Watt, 2004). Recently, anoikis was shown to be induced by the forced expression of αv integrin in an αv-negative SCC line,

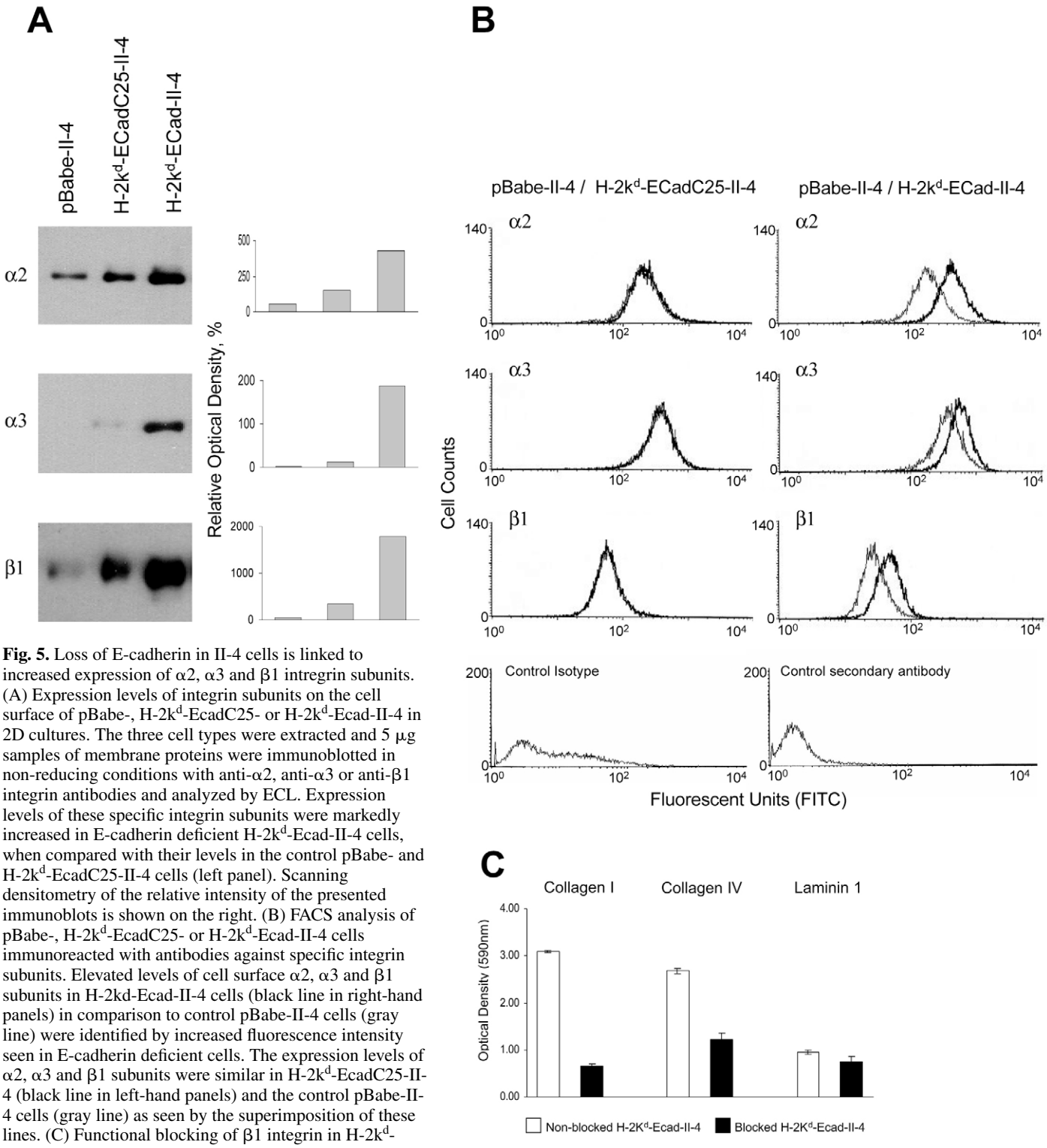

Fig. 5. Loss of E-cadherin in II-4 cells is linked to increased expression of α2, α3 and β1 intregrin subunits. (A) Expression levels of integrin subunits on the cell surface of pBabe-, H-2k^d-EcadC25- or H-2k^d-Ecad-II-4 in 2D cultures. The three cell types were extracted and 5 μg samples of membrane proteins were immunoblotted in non-reducing conditions with anti-α2, anti-α3 or anti-β1 integrin antibodies and analyzed by ECL. Expression levels of these specific integrin subunits were markedly increased in E-cadherin deficient H-2k^d-Ecad-II-4 cells, when compared with their levels in the control pBabe- and H-2k^d-EcadC25-II-4 cells (left panel). Scanning densitometry of the relative intensity of the presented immunoblots is shown on the right. (B) FACS analysis of pBabe-, H-2k^d-EcadC25- or H-2k^d-Ecad-II-4 cells immunoreacted with antibodies against specific integrin subunits. Elevated levels of cell surface α2, α3 and β1 subunits in H-2kd-Ecad-II-4 cells (black line in right-hand panels) in comparison to control pBabe-II-4 cells (gray line) were identified by increased fluorescence intensity seen in E-cadherin deficient cells. The expression levels of α2, α3 and β1 subunits were similar in H-2k^d-EcadC25-II-4 (black line in left-hand panels) and the control pBabe-II-4 cells (gray line) as seen by the superimposition of these lines. (C) Functional blocking of β1 integrin in H-2k^d-Ecad-II-4 cells. Two-dimensional cultures of Ecad-deficient H-2k^d-Ecad-II-4 cells were trypsinized briefly and replated for 15 minutes onto Type I and IV collagens or laminin-1 substrates, in the presence or absence of β1-integrin-blocking antibodies. Attached cells were quantified by optical density determined at 590 nm. Results are calculated as the mean ± s.d. of four replicates and experiments were repeated three times. $P<0.001$ for cell adherence to Type I collagen, Type IV collagen and laminin-1 substrates compared with levels in blocked controls.

demonstrating that levels of integrin subunit expression could modulate anoikis susceptibility and survival signaling (Janes and Watt, 2004). Interestingly, previous work also demonstrated that E-cadherin-mediated adhesion can counteract the requirement for matrix adhesion for epithelial cell survival thus enabling cells to circumvent anoikis (Kim et al., 1999; Green et al., 2004). These observations raise the question as to how loss of E-cadherin function could actually enhance survival and invasion of tumor cells in 3D tissue context as described in the present study. To reconcile these seemingly conflicting results we propose a dual role for E-cadherin in epithelial cell survival. When present in normal stratified squamous epithelial cells, E-cadherin may provide a physiological mechanism to counteract apoptosis induced by loss of ECM attachment as cells transit through suprabasal strata. However, when E-cadherin expression and/or function is lost during the early stages of epithelial neoplasia, small numbers of IE tumor cells are able to gain increased adhesion to extracellular matrix components at the BM, thus offsetting the loss of E-cadherin-mediated survival signals. Increased adhesion to the BM thus leads to intraepithelial retention of tumor cells at a site in the tissue that can subsequently facilitate initiation of tumor cell invasion.

The II-4 cell line used in our studies has been well characterized and represents an early stage of the malignant transformation process (Boukamp et al., 1990). Since II-4 cells harbor many of the important genetic hallmarks of the premalignant and early, invasive stages of SCC, such as mutations in the p53 gene and activation of Ha-Ras, this cell line is optimal for incorporation into models of early carcinoma progression. It is also important to understand if this genetic background contributes to the acquisition of adhesive and invasive properties seen upon loss of E-cadherin function in these cells. It is known that oncogenic Ha-Ras signaling cooperates with TGF-β to cause epithelial-mesenchymal transition (EMT) (Janda et al., 2002), that is a crucial event in late-stage tumorigenesis of transformed epithelial cells (Mercer et al., 2000). However, in early-stage tumor cells such as II-4, it is thought that mutation of the Ha-Ras gene may be more directly related to growth-regulatory pathways (Delhedde, 1999) rather than altering cell adhesion. As recently shown, E-cadherin-deficient-II-4 cells do not show all features of EMT (Margulis et al., 2005a), suggesting that the combinatorial effect of Ha-Ras activation, p53 loss and E-cadherin suppression are insufficient to fully induce EMT in these early-stage tumor cells.

While highlighting a novel aspect of E-cadherin loss in epithelial tumor progression, the current study extends our previous observations that loss of E-cadherin induces a highly aggressive tumor behavior in pure cultures of E-cadherin deficient cells (Margulis et al., 2005b) to mixtures of these cells with HEK. Only when the behavior of these cells was studied in the context of HEK has it been possible to determine that suppression of E-cadherin expression can induce loss of microenvironmental control of the malignant phenotype in II-4 cells in 3D tissues that mimic human premalignant disease. Similar impediments to tumor development inherent in 3D tissue context have been described in other tissue types, such as breast and prostate. For example, β1-integrin-mediated interactions between a premalignant breast epithelial cell line and adjacent ECM proteins have been shown to revert the malignant phenotype in a 3D model of early breast cancer progression (Miranti and Brugge, 2002; Wang et al., 1998; Schmeichel et al., 1998) by normalizing the distribution of E-cadherin upon reversion of malignant mammary epithelial cells to a normal phenotype (Weaver et al., 1997). Collectively, these observations demonstrate that genetic alterations present in individual initiated cells with malignant potential can have important functional consequences beyond tumor-autonomous roles in regulating cell cycle progression or cell survival. Rather, as in the case of E-cadherin loss, they significantly affect the complex interplay of tumor cells with the environment they find themselves in (Bissell and Radisky, 2001).

Our findings show that the integration of changes in cell-cell and cell-matrix adhesion are central to the conversion from premalignant lesions to early invasive carcinoma. These findings offer insight into incipient cancer invasion by demonstrating that microenvironmental, selective pressure drives the progression of precancer to fully-invasive tumors (Alt-Holland et al., 2005). This may help to explain why premalignant lesions such as actinic keratosis of skin, cervical dysplasia, oral leukoplakia and lobular carcinoma of the breast may contain numerous clones of initiated or dysplastic cells, yet have an unpredictable biological behavior that does not always advance to invasive cancer. By further understanding the progression of precancer to malignancy and by exploring signaling pathways associated with this transition in human 3D in-vivo-like tissue constructs, new therapeutic modalities designed to abrogate these events may be formulated to block early cancer invasion and thus prevent cancer occurrence.

Materials and Methods

2D cell culture

Human epidermal keratinocytes (HEK) were cultured from infant foreskin and were grown on an irradiated 3T3 fibroblast feeder layer, in DMEM containing 10% fetal calf serum (FCS). HaCaT-ras-II-4 (II-4) keratinocytes (Boukamp et al., 1990) that constitutively expressed the β-galactosidase gene to allow their identification in the context of HEK, were grown in DMEM containing 5% FCS. Two-dimensional, monolayer cultures of human dermal fibroblasts used for organotypic cultures were derived from infant foreskin and grown in media containing 10% FCS.

Retroviral infection

The dominant-negative retroviral E-cadherin vector (H-2k^{d}-Ecad) was a chimeric protein bearing the extracellular domain of mouse MHC class I antigen H-2K^{d} linked to the transmembrane domain of mouse E-cadherin (Zhu and Watt, 1996). Control vectors included H-2K^{d}-EcadC25, constructed from the H-2k^{d}-E-cad vector with a 25 amino acid deletion in the β-catenin-binding domain and the empty retroviral vector pBabe. The 293 Phoenix retroviral producer cells maintained in DMEM containing 10% bovine calf serum were transfected with pBabe, H-2K^{d}-Ecad and H-2K^{d}-EcadC25 plasmids (courtesy of F. Watt, Imperial Cancer Research Center, London, United Kingdom) by the calcium phosphate method.

3D cell culture and construction of premalignant tissues

Human tissue 3D constructs were prepared as previously described (Kolodka et al., 1998). HEK and II-4 cells that expressed each of the three retroviral constructs were mixed at 4:1 HEK:II-4 and 5×10^{5} cells from each cell mixture were seeded onto contracted collagen gels. Cultures were maintained submerged in low-calcium epidermal growth media for 2 days, submerged for 2 days in normal calcium epidermal growth media and raised to an air liquid interface for 5 days.

Transplantation of 3D cultures to nude mice

Three-dimensional tissue constructs were transplanted to the dorsum of 6-week-old male Swiss nude mice (N:NIHS-nuf DF; Taconic farms, Germantown, NY) and animals were sacrificed 4 weeks after transplantation. For routine light microscopy, tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin and 4 μm sections were stained with Hematoxylin and Eosin (H&E). Animal experiments were performed with the approval of the State University of New York (SUNY) Stony Brook Institutional Animal Care and Use Committees (IACUC).

Immunofluorescence staining

Tissue specimens were frozen in liquid nitrogen vapor and 6 μm serial sections were

mounted onto glass slides. Double immunofluorescent stains were performed using rabbit anti-β-gal (Cortex pharmaceuticals, CA) to detect II-4 cells and mouse anti-β-catenin (Zymed, CA) or mouse anti-Type IV collagen (Sigma) antibodies and detected using FITC-conjugated goat anti-rabbit IgG (Vector laboratories, CA) and Alexa Fluor 594-conjugated goat anti-mouse IgG (Molecular Probes, OR) antibodies. Fluorescence was visualized using a Nikon OptiPhot microscope.

Cell-matrix adhesion and functional blocking assays

Twenty-four-well plates coated with either Type I collagen, Type IV collagen, laminin, or fibronectin (Becton Dickinson, MA) were rinsed with PBS, blocked with 2% heat-denatured BSA at room temperature for 1 hour and rinsed again with PBS. II-4 cells expressing either of the E-cadherin vectors, or containing the pBabe-control vector, were trypsinized and resuspended in serum-free DMEM. Cells (5×10^4) were plated into each well, and plates were incubated at 37°C for 20 minutes. Cells were fixed at room temperature in 2% glutaraldehyde and non-adherent cells were removed by rinsing the plates several times in double-distilled H_2O. Plates were air-dried and adherent cells were stained with 0.1% crystal violet in 200 mM boric acid for 20 minutes. Plates were rinsed, air-dried and 10% acetic acid was added to each well. Optical density was determined at 590 nm.

For functional antibody blocking studies, cells were trypsinized, washed, resuspended in serum-free DMEM and preincubated for 45 minutes at 37°C in the absence (control, untreated cells) or the presence of 20 μg/ml of mouse anti-human β1-integrin function-blocking mAb (clone P5D2, Chemicon, Temecula, CA). Untreated II-4 cells or β1 integrin blocked II-4 cells (5×10^4 of each) were plated into each well and incubated at 37°C for 15 minutes. Thereafter, the procedure for fixing the cells, staining adherent cells and determination of optical density was the same as described above for adhesion assay.

Cell membrane fractionation and western blot analysis

Monolayer 2D cultures were extracted on ice into 400 μl cold PBS buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 10 mM NaF, 10 μg/ml aprotinin and leupeptin, 2 μg/ml pepstatin, 1 mM PMSF and 200 μl $NaVO_4$ (Sigma, St. Louis, MO). Cell lysates were subjected to five freeze-thaw cycles, centrifuged at 16,000 ***g*** for 25 minutes at 4°C and pellets were resuspended in 60 μl PBS containing 1% Triton X-100 as well as proteinase and phosphatase inhibitors. Pellets were homogenized four times over a 30 minute incubation on ice and centrifuged at 16,000 ***g*** for 25 minutes at 4°C. Protein concentration of the supernatant membrane fraction was measured using a modified Lowry assay (Bio-Rad DC Protein Assay Kit). For integrin analysis, 5 μg protein samples were boiled in Laemmli sample buffer without 2-β-Me, loaded onto 7.5% SDS-PAGE gel and separated proteins were transferred to a nitrocellulose membrane (Bio-Rad). Immunoblotting was performed using rabbit anti-human integrin α2, rabbit anti-human integrin α3 or mouse anti-human β1 integrin antibodies (Chemicon) following by HRP-anti-rabbit or HRP-anti-mouse IgG antibodies (Amersham, Piscataway, NJ). Protein bands were visualized by ECL, utilizing Pierce SuperSignal Kit (Rockford, IL).

Flow cytometry analysis of cell surface integrins

II-4 cells expressing either of the E-cadherin vectors or the pBabe-control vector were trypsinized, washed in serum-containing medium, and rinsed twice in cold PBS. Cells were resuspended and incubated for 30 minutes on ice in cold PBS with either mouse anti-human integrin α2, integrin α3 or integrin β1 antibodies (Chemicon). Cells were then washed twice in cold PBS and incubated on ice for 30 minutes with goat-anti mouse FITC-conjugated secondary antibody. Following extensive washing with cold PBS, cells were resuspended in PBS containing propidium iodide and live cells analyzed by the cell-sorter FACS Vantage (Becton-Dickinson Immunocytometry Systems, Mountain View, CA). Control cell samples for analyzing the background levels of immunostaining were incubated with either secondary antibody alone or unrelated mouse IgG and were used as negative controls.

We thank F. Watt for the gift of the chimeric, E-cadherin retroviral vectors. In addition, we would like to thank Ning Lin, Padmaja Prabhu, Marc Mendelsohn, Laura Bertolotti, Jennifer Landmann, Lorne Taichman and Larry Pfeiffer for their assistance. This work was supported by grant #2RO1DE011250-06 from the National Institutes of Dental and Craniofacial Research.

References

Alt-Holland, A., Zhang, W., Margulis, A. and Garlick, J. A. (2005). Microenvironmental control of premalignant disease. The role of intercellular adhesion in the progression of intraepithelial neoplasia. *Semin. Cancer Res.* **15**, 84-96.

Andriani, F., Garfield, J., Fusenig, N. E. and Garlick, J. A. (2004). Basement membrane proteins promote progression of intraepithelial neoplasia in 3-dimensional models of human stratified epithelium. *Int. J. Cancer* **108**, 348-357.

Avizienyte, E., Wyke, A. W., Jones, R. J., McLean, G. W., Westhoff, M. A., Brunton, V. G. and Frame, M. C. (2002). Src-induced de-regulation of E-cadherin in colon cancer cells requires integrin signalling. *Nat. Cell Biol.* **4**, 632-638.

Birchmeier, W. and Behrens, J. (1994). Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. *Biochim. Biophys. Acta* **1198**, 11-26.

Bissell, M. J. and Radisky, D. (2001). Putting tumors in context. *Nat. Rev. Cancer* **1**, 46-54.

Boukamp, P., Stanbridge, E. J., Foo, D. Y., Cerutti, P. A. and Fusenig, N. E. (1990). c-Ha-ras oncogene expression in immortalized human keratinocytes (HaCaT) alters growth potential in vivo but lacks correlation with malignancy. *Cancer Res.* **50**, 2840-2847.

Cano, A., Perez-Moreno, M. A., Rodrigo, I., Locascio, A., Blanco, M. J., del Barrio, M. G., Portillo, F. and Nieto, M. A. (2000). The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat. Cell Biol.* **2**, 76-83.

Cavallaro, U. and Christofori, G. (2004). Call adhesion and signaling by cadherins and Ig-CAMs in cancer. *Nat. Rev. Cancer* **4**, 118-132.

Delhedde, M., Song, H. C., Hamm, R., Brisbay, S., Ananthaswamy, H. N., Kripke, M. and McDonnell, T. J. (2001). Impact of Bcl-2 and Ha Ras on keratinocytes in organotypic culture. *J. Invest. Dermatol.* **116**, 366-373.

Dlugosz, A., Merlino, G. and Yuspa, S. H. (2002). Progress in cutaneous cancer research. *J. Invest. Dermatol. Symp. Proc.* **7**, 17-26.

Friedl, P. and Wolf, K. (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat. Rev. Cancer* **3**, 362-374.

Frisch, S. M. and Francis, H. (1994). Disruption of epithelial cell-matrix interactions induces apoptosis. *J. Cell Biol.* **124**, 619-626.

Gotzmann, J., Mikula, M., Eger, A., Schulte-Hermann, R., Foisner, R., Beug, H. andMikulits, W. (2004). Molecular aspects of epithelial cell plasticity: implications for local tumor invasion and metastasis. *Mut. Res.* **566**, 9-20.

Green, S. K., Francia, G., Isidoro, C. and Kerbel, R. S. (2004). Antiadhesive antibodies targeting E-cadherin sensitize multicellular tumor spheroids to chemotherapy in vitro. *Mol. Cancer Ther.* **3**, 149-159.

Jamora, C. and Fuchs, E. (2002). Intercellular adhesion, signalling and the cytoskeleton. *Nat. Cell Biol.* **4**, E101-E108.

Janda, E., Lehmann, K., Killisch, I., Jechlinger, M., Herzig, M., Downward, J., Beug, H. and Grunert, S. (2002). Ras and TGF [beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. *J. Cell Biol.* **156**, 299-313.

Janes, S. M. and Watt, F. M. (2004). Switch from alphavbeta5 to alphavbeta6 integrin expression protects squamous cell carcinomas from anoikis. *J. Cell Biol.* **166**, 419-431.

Javaherian, A., Vaccariello, M., Fusenig, N. E. and Garlick, J. A. (1998). Normal keratinocytes suppress early stages of neoplastic progression in stratified epithelium. *Cancer Res.* **58**, 2200-2208.

Jost, M., Kar, C. and Rodeck, U. (2001). Matrix-independent survival of human keratinocytes through activation of the EGF receptor. *Mol. Biol. Cell* **12**, 1519-1527.

Karen, J., Wang, Y., Javaherian, A., Vaccariello, M., Fusenig, N. E. and Garlick, J. A. (1999). 12-O-tetradecanoylphorbol-13-acetate induces clonal expansion of potentially malignant keratinocytes in a tissue model of early neoplastic progression. *Cancer Res.* **59**, 474-481.

Kim, W. H., Lee, B. L., Kim, D. K. and Kleinman, H. K. (1999). Laminin-1-adherent cancer cells show increased proliferation and decreased apoptosis in vivo. *Anticancer Res.* **19**, 3067-3071.

Kolodka, T. M., Garlick, J. A. and Taichman, L. B. (1998). Evidence for keratinocyte stem cells in vitro: long term engraftment and persistence of transgene expression from retrovirus-transduced keratinocytes. *Proc. Natl. Acad. Sci. USA* **95**, 4356-4361.

Margulis, A., Zhang, W., Alt-Holland, A., Pawagi, S., Pfieffer, L., Garfield, J., Fusenig, N. E. and Garlick, J. A. (2005a). Loss of intercellular adhesion activates a transition from low- to high-grade human squamous cell carcinoma. *Int. J. Cancer* doi:10.1002/ijs.21409.

Margulis, M., Zhang, W., Alt-Holland, A., Crawford, H., Fusenig, N. E. and Garlick, J. A. (2005b). E-cadherin suppression accelerates squamous cell carcinoma progression in 3D human tissue constructs *Cancer Res.* **65**, 1783-1791.

Mercer, J. A. (2000). Intercellular junctions: downstream and upstream of Ras? *Semin. Cell Dev. Biol.* **11**, 309-314.

Meredith, J. E., Jr, Fazeli, B. and Schwartz, M. A. (1993). The extracellular matrix as a cell survival factor. *Mol. Biol. Cell* **4**, 953-961.

Miranti, C. K. and Brugge, J. S. (2002). Sensing the environment: a historical perspective on integrin signal transduction. *Nat. Cell Biol.* **4**, E83-E90.

Mudgil, A. V., Segal, N., Andriani, F., Wang, Y., Fusenig, N. E. and Garlick, J. A. (2003). Ultraviolet B irradiation induces expansion of intraepithelial tumor cells in a tissue model of early cancer progression. *J. Invest. Dermatol.* **121**, 191-197.

Perl, A. K., Wilgenbus, P., Dahl, U., Semb, H. and Christofori, G. (1998). A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature* **392**, 190-193.

Schmeichel, K. L., Weaver, V. M. and Bissell, M. J. (1998). Structural cues from the tissue microenvironment are essential determinants of the human mammary epithelial cell phenotype. *J. Mammary. Gland Biol. Neoplasia* **3**, 201-213.

Vaccariello, M., Javaherian, A., Wang, Y., Fusenig, N. E. and Garlick, J. A. (1999). Cell interactions control the fate of malignant keratinocytes in an organotypic model of early neoplasia. *J. Invest. Dermatol.* **113**, 384-391.

von Schlippe, M., Marshall, J. F., Perry, P., Stone, M., Zhu, A. J. and Hart, I. R. (2000). Functional interaction between E-cadherin and alphav-containing integrins in carcinoma cells. *J. Cell Sci.* **113**, 425-437.

Wang, F., Weaver, V. M., Petersen, O. W., Larabell, C. A., Dedhar, S., Briand, P., Lupu, R. and Bissell, M. J. (1998). Reciprocal interactions between β1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology. *Proc. Natl. Acad. Sci. USA* **95**, 14821-14826.

Weaver, V. M., Petersen, O. W., Wang, F., Larabell, C. A., Briand, P., Damsky, C. and Bissell, M. J. (1997). Reversion of the malignant phenotype of human breast cells in three-dimensional culture and *in vivo* by integrin blocking antibodies. *J. Cell Biol.* **137**, 231-245.

Wu, C. (1999). Integrin-linked kinase and PINCH: partners in regulation of cell-extracellular matrix interaction and signal transduction. *J. Cell Sci.* **112**, 4485-4489.

Yano, H., Mazaki, Y., Kurokawa, K., Hanks, S. K., Matsuda, M. and Sabe, H. (2004). Roles played by a subset of integrin signaling molecules in cadherin-based cell-cell adhesion. *J. Cell Biol.* **166**, 283-295.

Zhang, F., Tom, C. C., Kugler, M. C., Ching, T. T., Kreidberg, J. A., Wei, Y. and Chapman, H. A. (2003). Distinct ligand binding sites in integrin alpha3beta1 regulate matrix adhesion and cell-cell contact. *J. Cell Biol.* **163**, 177-188.

Zhu, A. J. and Watt, F. M. (1996). Expression of a dominant negative cadherin mutant inhibits proliferation and stimulates terminal differentiation of human epidermal keratinocytes. *J. Cell Sci.* **109**, 3013-3023.

Malignant Transformation of Immortalized HaCaT Keratinocytes through Deregulated Nuclear Factor κB Signaling

Qing Ren,[1] Csaba Kari,[2] Marlene R.D. Quadros,[2] Randy Burd,[1] Peter McCue,[3] Adam P. Dicker,[1] and Ulrich Rodeck[2]

Departments of [1]Radiation Oncology, [2]Dermatology and Cutaneous Biology, and [3]Pathology, Thomas Jefferson University, Philadelphia, Pennsylvania

Abstract

Previous studies addressing functional aspects of nuclear factor κB (NF-κB) activation in normal and transformed keratinocytes revealed complex and seemingly contradictory roles of this transcription factor in this cell type. In normal skin, NF-κB signaling seems to inhibit squamous cell carcinoma development whereas, in squamous cell carcinoma themselves, deregulated NF-κB expression and/or signaling is frequently observed. To further investigate this paradox, we focused on NF-κB activation as it relates to the transformed phenotype of immortalized but nontumorigenic human keratinocytes (HaCaT cells). We observed that NF-κB activity contributed to survival and growth of cultured HaCaT keratinocytes as shown by use of pharmacologic NF-κB inhibitors, RNA interference, and inducible overexpression of a dominant interfering IκB construct. NF-κB activation was largely provided through interaction with extracellular matrix components because preventing cell attachment by forced suspension culture markedly reduced NFκB signaling associated with cell death (anoikis); conversely, anoikis was partially reversed by NF-κB activation induced either by tumor necrosis factor-α treatment or by overexpressing the NF-κB p65 subunit in HaCaT cells. Furthermore, overexpression of NF-κBp65 in HaCaT cells induced colony formation in soft agar and tumorigenicity in nude mice. In summary, as opposed to normal keratinocytes, immortalized HaCaT keratinocytes provide a cellular context in which deregulated NF-κB signaling supports multiple malignant traits *in vitro* and *in vivo*. (Cancer Res 2006; 66(10): 5209-15)

Introduction

The transcription factor nuclear factor κB (NF-κB) was first identified as a nuclear factor in B lymphocytes, which binds to the enhancer of the immunoglobulin κ light chain. The NF-κB family contains several members including Rel A (p65), Rel B, c-Rel, p50 (NF-κB1), and p52 (NF-κB2), which exist as homodimers and heterodimers retained by ankyrin domain–containing IκBs in the cytoplasm of unstimulated cells (for review, see ref. 1). On stimulation of cells with inflammatory cytokines, IκB is phosphorylated by IκB kinases, ubiquitinated, and proteasomally degraded. Dissociation from IκB enables nuclear translocation of NF-κB dimers where they direct transcription of a host of target genes, many of which encode antiapoptotic proteins. In addition to their roles in immune and inflammatory processes, NF-κB family members have been observed to have oncogenic properties (2). Consistent with important roles of NF-κB in tumorigenesis, enhanced NF-κB activity has been observed in malignant tumors of diverse origin including carcinomas (for review, see ref. 3).

Investigations into the role of NF-κB in normal epidermis and the development of squamous cell carcinomas of the skin or mucous membranes revealed complex and seemingly contradictory roles of this transcription factor. As in other epithelial malignancies, deregulated NF-κB signaling is well documented in squamous cell carcinomas (4–6). Yet, when NF-κB signaling is disrupted in normal epidermis, squamous cell carcinomas develop at increased rates (7, 8). The present study was undertaken to probe the relevance of NF-κB signaling to the malignant phenotype of nontumorigenic human epidermal keratinocytes representing early stages of malignant transformation (HaCaT; ref. 9). We describe that NF-κB activation through interaction with extracellular matrix components provided an important survival mechanism to HaCaT cells that was disrupted by preventing cell attachment to extracellular matrix by forced suspension culture. Reduced NF-κB signaling and cell death in forced suspension culture could be partially reversed by tumor necrosis factor-α (TNFα)–induced NF-κB activation. Deregulated NF-κB signaling achieved by overexpressing the NF-κB p65 subunit similarly increased survival of HaCaT cells in forced suspension culture, induced colony formation in soft agar, and resulted in tumorigenicity of HaCaT cells in nude mice. These observations underscore that molecular alterations incurred during immortalization of HaCaT keratinocytes have conferred oncogenic properties to deregulated NF-κB signaling. This is in clear contrast to normal keratinocytes in which NF-κB activation inhibits proliferation and reduces the propensity of malignant transformation (7, 10, 11).

Materials and Methods

Cell lines and culture conditions. Immortalized human keratinocytes (HaCaT) and derivative cells were maintained in culture medium (W489) supplemented with 2% FCS as previously described (12). HaCaTp65 cells stably expressing the COOH-terminally hemagglutinin-tagged human NF-κB p65 subunit were selected using 1 μg/mL puromycin. Selection of antibiotic-resistant cells started 48 hours after transfection with NF-κBp65-pIRESpuro2 vector (BD BioSciences, Palo Alto, CA) and continued until resistant colonies appeared. In this vector, both the NF-κBp65 gene and the puromycin resistance gene are expressed from one bicistronic mRNA, in which the internal ribosomal entry site of the encephalomyocarditis virus permits translation of the puromycin *N*-acetyltransferase coding region downstream of the NF-κBp65 gene. Because all the surviving colonies

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Ulrich Rodeck, Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, 233 South 10th Street, BLSB 409, Philadelphia, PA 19107. Phone: 215-503-5622; Fax: 215-503-5622; E-mail: ulrich.rodeck@mail.tju.edu and Adam P. Dicker, Department of Radiation Oncology, Thomas Jefferson University, 111 South 11th Street, Philadelphia, PA 19107. Phone: 215-955-6527; Fax: 215-955-0412; E-mail: adam.dicker@mail.tju.edu.

doi:10.1158/0008-5472.CAN-05-4158

expressed the transgene, there was no need for clonal selection, but rather all the puromycin-resistant clones were pooled and cultured under selective condition. Using this protocol, individual clonal variations are eliminated. Two independent transfections and selections were made generating two separate NF-κBp65-overexpressing cell lines exhibiting similar phenotypic characteristics. The *IκBα* superrepressor gene tagged at the COOH terminus with hemagglutinin (Upstate Biotechnology, Lake Placid, NY) was cloned into the tetracycline regulatable episomal expression vector pCEPTetP as previously described by us (13). The vector was transfected into the HaCaT-tTA1 cell line expressing the tetracycline-controlled transactivator (13) and hygromycin-resistant colonies were pooled. Unless noted otherwise, all experiments were done in serum-free, growth factor–free KGM medium formulated for keratinocyte growth (13).

RNA interference. Small interfering RNA (siRNA) targeted to down-regulate NF-κBp65 mRNA (siGENOME SMARTpool siRNA, Dharmacon, Lafayette, CO) was transfected into HaCaT cells using RNAiFECT reagent (Qiagen, Valencia, CA) according to the protocol of the manufacturer. Nonsense siRNA provided by the manufacturer was used as a negative control. Cell lysates were collected at 24, 48, and 72 hours posttransfection.

Antibodies and immunoblot analyses. Immunoblot analyses were done using standard procedures as previously described by us (13). Antibodies to NF-κBp65, β-actin, and IκB (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used at 1 μg/mL. The antibody against α-tubulin (Calbiochem, San Diego, CA) was used at 1 μg/mL. The antibody against cleaved poly(ADP-ribose) polymerase (PARP; Cell Signaling, Beverly, MA) was used at 1:1,000 dilution. Goat anti-rabbit secondary antibody (IRDye 800CW, Rockland Inc., Gilbertsville, PA) and goat anti-mouse secondary antibody (Alexa Fluor 680, Molecular Probes, Eugene, OR) for Odyssey IR imaging were used at 1:10,000 and 1:2,000 dilutions, respectively. Signal analysis was done either by film exposure or digitally by scanning membranes with the Odyssey IR imaging system (Li-Cor Biosciences, Lincoln, NE).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Subconfluent HaCaT cells were trypsinized and reseeded onto cell culture–treated 96-well plates in serum-free, growth factor–free KGM in the presence and absence of NF-κB inhibitor Bay11-7082 (5 μmol/L; Biomol, Plymouth Meeting, PA). Culture medium was exchanged to fresh growth medium (W489 containing 2% FCS) 24 hours after seeding. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were done 3 days later by incubating cultures with MTT solution (Sigma, St. Louis, MO) at 37°C for 4 hours. MTT solubilization solution (10% Triton X-100/0.1 N HCL in isopropanol) was added and the tissue culture plates were incubated for additional 12 hours at 37°C before absorbance was spectrophotometrically assessed at 570 nm (reference wavelength, 690 nm).

Caspase-3 activity assay. For the caspase-3 substrate cleavage assay, cells were processed and assayed using the EnzCheck Caspase-3 Assay Kit (Molecular Probes). Fluorescence was measured at emission and excitation settings of 485/530 nm with a Bio-Tek FL600 plate reader (Winooski, VT).

Anchorage-independent survival assay. Anchorage-independent cell survival was assessed as previously described (13). Briefly, tissue culture plates were coated with 0.9% agarose in serum-free medium. HaCaT cells were seeded on top of the agarose layer in serum-free KGM (1.5 mL/well) at 2×10^5/mL. Twenty-four hours later, 600-μL aliquots of cell solution were transferred to cell culture–treated plastic and supplemented with fresh media (W489 with 2% FCS). After 24 hours, reattached cells were fixed with 70% ethanol for 5 minutes. Cells were stained with crystal violet solution (0.2% in methanol), rinsed with water, and air-dried.

NF-κB DNA binding assay. Subconfluent HaCaT cells were trypsinized and seeded in serum-free medium (6 mL; 4×10^5 cells/mL) in 100-mm plates precoated with 0.9% agarose with or without TNFα (1 μg/mL). Cells were collected for nuclear extract preparation after various times in forced suspension culture (4, 8, and 24 hours). For comparison, nuclear extracts were also prepared immediately after trypsinization of HaCaT cells. DNA binding of NF-κB was determined using the NF-κB p50/p65 Transcription Factor Assay (Chemicon International, Temecula, CA) according to the instructions of the manufacturer. Briefly, nuclear extracts were prepared and incubated with double-stranded biotinylated oligonucleotides containing consensus NF-κB binding sequences. NF-κB/DNA complexes were captured on 96-well plates coated with streptavidin. NF-κB bound to DNA was detected colorimetrically (450/650 nm) after sequential incubation with an antibody against NF-κBp65 and horseradish peroxidase–conjugated secondary antibody.

NF-κB reporter (secreted alkaline phosphatase) assay. Cells were seeded at 7.5×10^4/mL in KGM for 1 to 2 days before cotransfection with pSEAP2-NF-κB vector (BD BioSciences) encoding a secreted form of human placental alkaline phosphatase driven by a NF-κB-responsive promoter and a β-galactosidase expression vector (14) for control purposes. NF-κB-dependent transcription was determined 48 hours after transfection using the Great EscAPe SEAP Reporter System 3, which is based on detection of secreted alkaline phosphatase in cell supernatants normalized to β-galactosidase activity using the luminescent β-gal detection kit (BD Biosciences).

Soft agar colony formation assay. Growth and survival of HaCaT cell variants was determined as previously described (15). Briefly, cells growing in monolayer culture were trypsinized and resuspended (10^4 cells) in 2 mL medium containing 0.4% agar and 10% FCS. Cell suspensions were added to 0.6% agar layers in six-well plates. Wells examined immediately after plating showed only single cells. Soft agar colonies of >50 cells were scored after 14 days in triplicate.

Tumorigenicity of HaCaT variants in nude mice. HaCaT, HaCaTII-4, and HaCaTp65 single-cell suspensions were injected s.c. into the right hind limbs (5×10^6 for HaCaTII-4 and 1×10^7 for other cell lines in 100 μL PBS) of 8-week-old athymic NCR NUM mice (Taconic Farms, Hudson, NY). Tumor growth was monitored every 2 days for 2 months. Tumor volume was determined by direct measurement with calipers and calculated by the formula [(smallest diameter^2 × widest diameter) / 2]. Tumors that grew >500 mm^3 and did not regress were considered to be established tumors. Tumors were not allowed to grow beyond 2,000 mm^3 in accordance with Institutional Animal Care and Use Committee regulations.

Immunohistochemical analyses. Paraffin-embedded blocks containing the mouse tumor tissue were fixed in 10% neutral-buffered formalin and processed for H&E staining and immunohistochemical analysis. Tissue sections were deparaffinized in xylene, rehydrated in ethanol, rehydrated with water, and washed in 1% PBS. Primary antibodies (HA-Tag, Cell Signaling; NFκB, Santa Cruz Biotechnology) were applied to slides and incubated for 45 to 60 minutes. The immune complexes were visualized with the chromogenic substrate Dako Liquid DAB+ Substrate-Chromogen Solution (diaminobenzidine tetrahydrochloride, K3468, DAKO, Carpinteria, CA) for 5 minutes.

Results

NF-κB activation contributes to survival of immortalized keratinocytes in steady-state culture conditions. NF-κB signaling has recently been shown to support survival of immortalized mammary epithelial cells in three-dimensional tissue reconstructs (16, 17). Here we assessed whether NF-κB signaling similarly contributed to the survival of immortalized human epidermal keratinocytes. To this end, we first used the NF-κB inhibitor Bay11-7082 in immortalized human keratinocytes (HaCaT). Bay11-7082 efficiently decreased transcriptional NF-κB activity in HaCaT keratinocytes (Fig. 1*A*). Consistent with an important role of NF-κB in HaCaT cell proliferation or survival, we observed that, within 24 hours of Bay11-7082 addition, metabolic activity of HaCaT cells was markedly reduced as determined by MTT assay (Supplementary Fig. S1). Similar results were obtained when using other inhibitors of NF-κB activity (i.e., the proteasome inhibitor MG-132 and parthenolide; Supplementary Fig. S1). Inhibition of MTT conversion was associated with extensive membrane blebbing and nuclear condensation (not shown) and with p85PARP cleavage (Fig. 1*A*), consistent with caspase-3 activation and apoptotic cell death. To obtain independent evidence for a role of NF-κB in HaCaT cell survival, we assessed the effects of down-regulating the

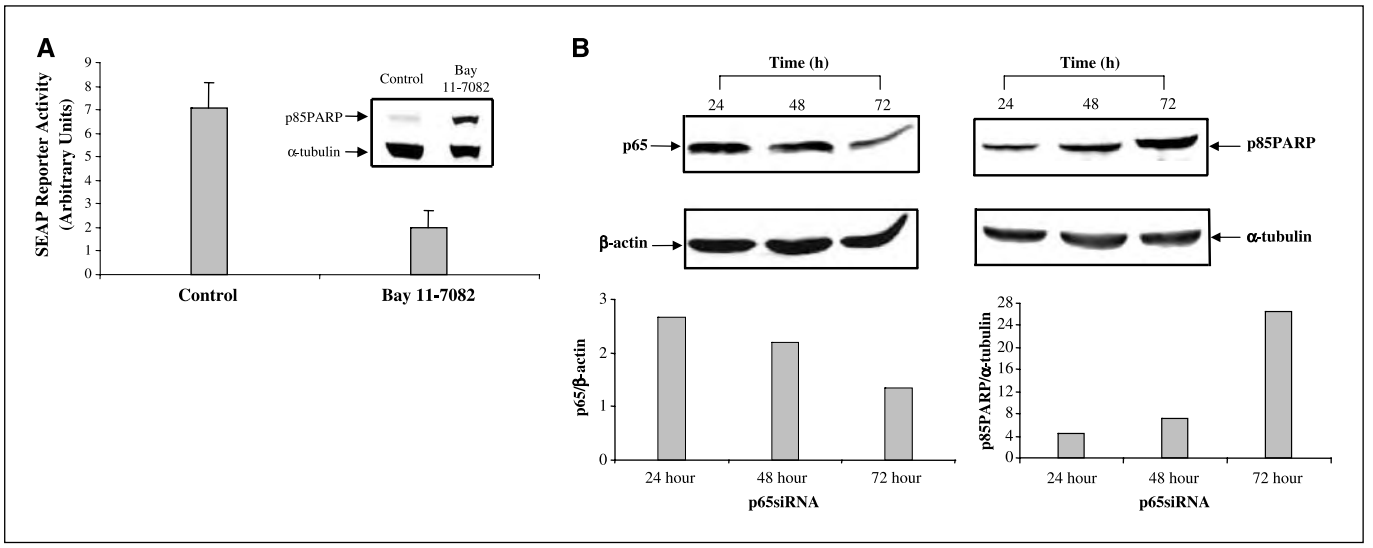

Figure 1. Induction of apoptosis in HaCaT keratinocytes by down-regulating NF-κB activity or expression. *A,* inhibition of NF-κB activity in HaCaT cells in monolayer culture treated with NF-κB inhibitor Bay11-7082 (5 μmol/L) for 24 hours as compared with controls that received vehicle only. NF-κB activity was determined using the Great EscAPe SEAP Reporter System 3 as described in Materials and Methods. *Columns,* mean of three independent experiments; *bars,* SD. $P < 0.05$, Student's *t* test. *Inset,* apoptosis induction in HaCaT cells by treatment with Bay11-7082. Cell extracts were prepared 24 hours after Bay11-7082 was added and caspase-3 activity assessed by detection of the caspase-3 cleavage product of PARP. For control purposes, blots were rehybridized with an antibody to α-tubulin. *B,* time-dependent changes in expression of NF-κBp65 and the p85PARP cleavage product in siRNA-treated cells. Densitometric representation of the immunoblot results relative to expression of β-actin or α-tubulin as indicated.

expression of the NF-κB p65 subunit in HaCaT cells by way of siRNA (Fig. 1*B*). As expected, transfection of NF-κBp65-targeted siRNA was associated with reduced expression of the NF-κBp65 protein and increased PARP cleavage. To further test the role of NF-κB activation in HaCaT cell survival, we transfected these cells with a mutant IκBα [IκB superrepressor (IκBSR)] which contains serine-to-alanine mutations at amino acids 32 and 36. The IκBSR construct is resistant to IKK-dependent phosphorylation, ubiquitination, and proteasomal degradation, and serves to inhibit NF-κB-dependent transactivation by sequestering NF-κB in the cytoplasm (18). Attempts at stable, constitutive expression of this construct failed (not shown), necessitating the use of a previously established inducible expression system (14). On removal of tetracycline from the medium, expression of the hemagglutinin-tagged IκBSR was efficiently induced (Fig. 2*A*). As expected, induced expression of the dominant interfering IκB resulted in markedly reduced NF-κB activity as assessed by transcription of a reporter gene driven by a NF-κB-responsive promoter (Fig. 2*B*). In addition, induced expression of IκBSR resulted in enhanced apoptosis as determined by assessment of caspase-3 activity in cell extracts collected at several time points after transgene induction (Fig. 2*C*). Of note, even in the noninduced state, slightly enhanced levels of caspase-3-dependent substrate cleavage were observed over time, perhaps due to leakiness of the expression construct. Taken together, these results underscore that NF-κB activation is an important requisite for survival of cultured HaCaT cells.

Extracellular matrix adhesion maintains NF-κB activation in HaCaT cells. Next, we asked whether matrix adhesion itself contributed to NF-κB activity under steady-state culture conditions. This investigation was prompted by the observation that maintaining HaCaT cells in the absence of exogenous growth factors had only marginal effects on NF-κB activity (not shown). To address this question, we placed HaCaT cells in forced suspension culture as previously described (13) and assayed NF-κB activity under those conditions for up to 24 hours. Consistent with an important role of matrix adhesion in maintaining NF-κB activity in HaCaT cells, we observed a continuous decrease in NF-κB activity over the observation period (Fig. 3*A*).

TNF-α treatment rescues NF-κB activity and enhances HaCaT cell survival in forced suspension culture. The results described above raised the question whether inflammatory cytokines known to stimulate NF-κB activity can supplant the requirement for extracellular matrix adhesion for NF-κB activation in the suspended state. Among such cytokines, TNF-α is one of the strongest NF-κB activators (1). We therefore determined whether TNF-α treatment counteracted the reduction of NF-κB activity incurred by HaCaT cells in forced suspension culture. Consistent with this notion, we observed attenuated reduction of NF-κB activity in suspended cells in the presence of TNF-α (Fig. 3*A*). This observation led us to assess the effect of TNF-α addition on survival of HaCaT cells in suspension culture. In agreement with an important role of NF-κB activity in HaCaT cell survival, rescue of NF-κB activity by TNF-α treatment was associated with enhanced survival of HaCaT cells in forced suspension culture (Fig. 3*B*). This was determined by assessing colony formation of HaCaT cells reseeded on cell culture–treated plastic after 24 hour of forced suspension culture. As compared with untreated controls, TNF-α treatment during suspension increased both the number and size of colonies. Specifically, the number of colonies in the presence of TNF-α was consistently increased by 40% whereas treatment with epidermal growth factor increased colony formation by 130% as described previously (Fig. 3*C*; ref. 13). As expected, the survival advantage conferred by TNF-α addition was drastically attenuated by addition of the NF-κB inhibitor Bay11-7082. Because TNF-α is known to induce apoptosis in certain cell systems and to avoid such proapoptotic effect of TNF-α, we initially included caspase 8 inhibitors in the experiments probing the effects of TNF-α on HaCaT cell survival in forced suspension. However, the inclusion of such inhibitors was not necessary for TNF-α to exert its survival effect in forced suspension cultures. Collectively, these results

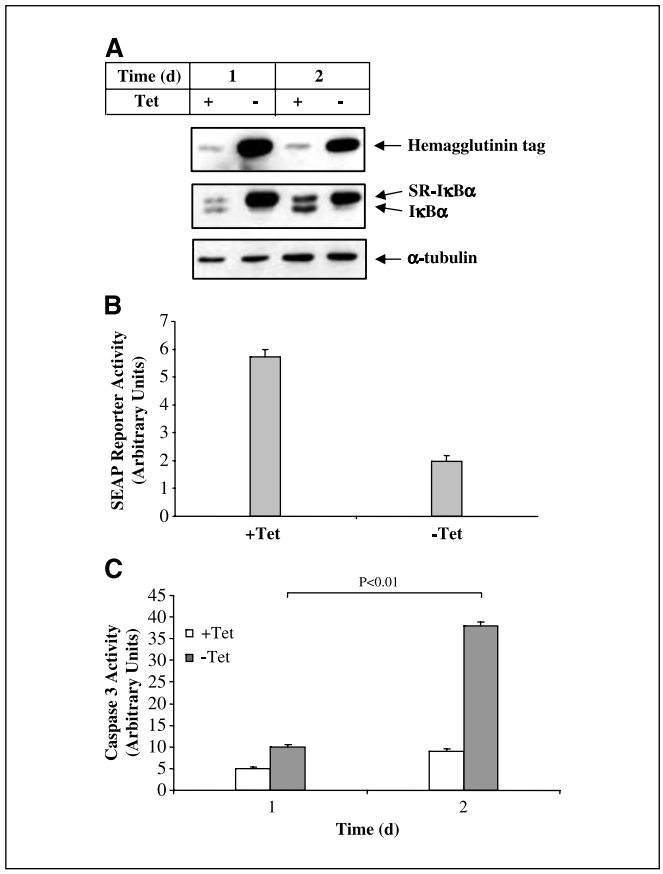

Figure 2. Inhibition of NF-κB activity by overexpressing of the IκBα superrepressor induces apoptosis in HaCaT cells. *A,* expression of the IκBα superrepressor was induced in HaCaT cells by removal of tetracycline from culture media. Transgene expression was monitored by Western blot analysis of the hemagglutinin tag and IκBαSR itself as indicated. *B,* NF-κB-dependent promoter activity in induced and uninduced HaCaT cells determined 24 hours after transgene induction using a reporter gene assay. *Columns,* mean of three independent experiments; *bars,* SD. $P < 0.01$, Student's *t* test. *C,* effect of IκBα superrepressor expression on HaCaT cells survival. Apoptosis induction by IκBα superrepressor expression is evident by measuring caspase-3 activity using a fluorogenic substrate as described in Materials and Methods.

assigned an unexpected new role to TNF-α in support of anchorage-independent epithelial cell survival.

Deregulated NF-κB signaling supports anchorage-independent HaCaT cell survival. To independently test whether TNF-α-mediated HaCaT cell survival in forced suspension culture could be accomplished through up-regulating NF-κB activity, we established HaCaT cells stably expressing the NF-κB p65 subunit (Fig. 4*A*). As expected, these cells revealed increased constitutive NF-κB activity relative to mock-transfected cells. Furthermore, when subjected to forced suspension culture, these cells survived in markedly higher numbers when compared with controls (Fig. 4*B*). TNF-α treatment did not further enhance the rescue effect observed in NF-κBp65-expressing HaCaT cells, in agreement with the notion that the TNF-α effect on HaCaT cell survival is primarily due to activation of NF-κB. Finally, and as expected, Bay11-7082 treatment drastically attenuated NF-κB activity (Supplementary Fig. S3) and survival of NF-κBp65 HaCaT cells in suspension culture (Fig. 4*B*).

Overexpression of NF-κBp65 induces soft agar colony formation by HaCaT cells. The observation that deregulated NF-κB activity supported anchorage-independent survival of HaCaT cells in liquid culture encouraged us to ascertain whether overexpression of NF-κBp65 also affected colony formation in soft agar, an important parameter of advanced malignancy in cells derived from solid tumors. This analysis revealed that, consistent with earlier results (9, 19), mock-transfected HaCaT cells did not form soft agar colonies (Fig. 5). In contrast, NF-κBp65-overexpressing HaCaT cells formed colonies, albeit to a lesser degree, than fully transformed Ha-RasV12-expressing HaCaTII-4 cells included as a positive control (19). This experiment was repeated with a second, independently derived p65-overexpressing HaCaT transfectant which also formed colonies in soft agar (data not shown).

Overexpression of NF-κBp65 induces tumor formation of HaCaT cells in nude mice. Finally, we addressed the question whether NF-κBp65 overexpression rendered HaCaT cells tumorigenic in immunodeficient mice. HaCaT cells do not form tumors when xenotransplanted to immunodeficient nude mice (19). In marked contrast, we observed that HaCaTp65 cells formed tumors in all six mice injected with 1×10^7 cells per mouse (Fig. 6) whereas mock-transfected control HaCaT cells did not. Expression of the NF-κBp65 transgene in tumor tissues was confirmed in tumor cells *in situ* by immunostaining using antibodies to both the

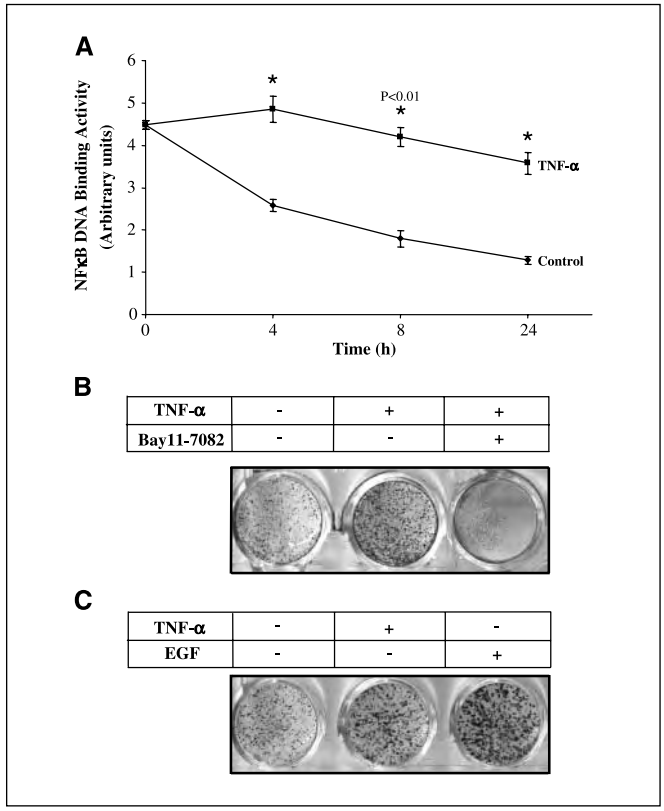

Figure 3. TNF-α enhances HaCaT cell survival in forced suspension culture through activation of NF-κB. *A,* HaCaT cells were suspended in plates precoated with 0.9% agarose in serum-free medium. NF-κB activity was evaluated in cell extracts prepared at the time points indicated by assessing DNA binding of the active form of NF-κB in nuclear extracts as described in Materials and Methods. Treatment of suspended cells with TNF-α (10 ng/mL) counteracted the gradual loss of NF-κB DNA binding activity observed in control cells. *B,* HaCaT cells were suspended in plates precoated with 0.9% agarose with or without TNF-α (10 ng/mL) and the NF-κB inhibitor Bay11-7082 (5 μmol/L) as indicated for 24 hours. Aliquots of cells were reseeded in fresh medium on tissue culture–treated plastic. Crystal violet staining of reattached viable cells was done 24 hours later. *C,* comparison of the effects of EGF (10 ng/mL) and of TNF-α (10 ng/mL) on HaCaT cell survival in forced suspension cultures.

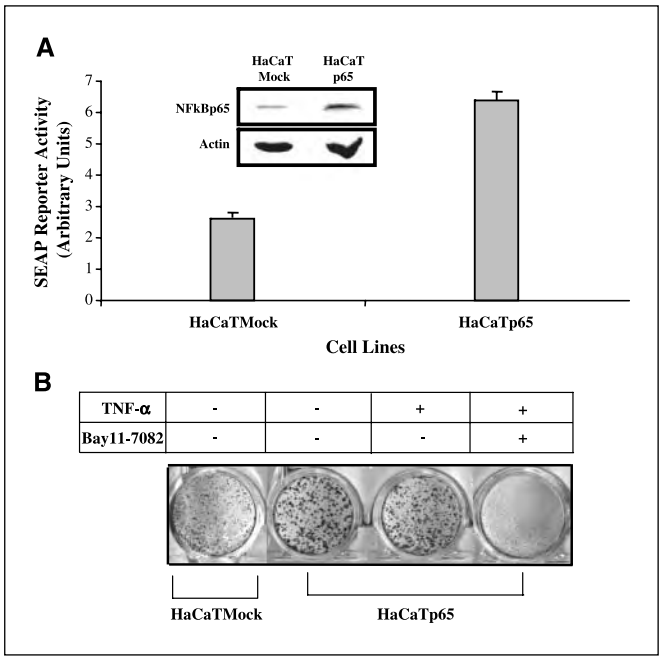

Figure 4. Overexpression of the NF-κB p65 subunit supports HaCaT cell survival in forced suspension culture. *A,* NF-κB promoter activity in HaCaT cells stably transfected with NF-κBp65 (HaCaTp65) as compared with parental HaCaT cells. *Columns,* mean of three independent experiments; *bars,* SD. $P < 0.01$, Student's *t* test. *Inset,* expression levels of NF-κBp65 in parental HaCaT and HaCaTp65 cells. *B,* enhanced survival in forced suspension culture of HaCaTp65 cells. Cells reseeded after 24 hours of forced suspension culture. Note that survival of HaCaTp65 cells was not further stimulated by TNF-α treatment and was markedly inhibited by NF-κB inhibitor Bay11-7082.

hemagglutinin tag and NF-κBp65 itself (Supplementary Fig. S2). To confirm that NF-κBp65 overexpression in HaCaT cells induces tumorigenicity in these cells, we used a second, independently derived p65-overexpressing HaCaT cell variant which also formed tumors (not shown). In these experiments, we used Ha-Ras-transformed HaCaTII-4 cells as a positive control; in contrast to HaCaT cells, HaCaTII-4 cells have previously been described to be tumorigenic in nude mice when injected at 5×10^6 per mouse (19). We confirmed these earlier findings for both HaCaT and HaCaTII-4 cells, which were nontumorigenic and tumorigenic, respectively. HaCaTII-4 cells formed tumors in three of six inoculated mice. HaCaTII-4 tumors presented as moderate to poorly differentiated squamous cell carcinomas (Fig. 6). These tumors grew in broad sheets and without significant keratin production. Numerous mitotic figures were present and cell borders were well delineated with intercellular bridges. By contrast, HaCaTp65 tumors represented well-differentiated squamous cell carcinomas with abundant eosinophilic cytoplasm, consistent with keratin production. In comparison with HaCaTII-4 tumors, only rare mitotic figures were present in HaCaTp65 tumors along the periphery of the neoplastic lesions. In addition, neutrophils and, to a lesser extent, lymphocytes were prominent within the tumor and at the tumor/stromal interface. Collectively, these data revealed that overexpression of NF-κBp65 in HaCaT cells was associated with tumor formation *in vivo* and with inflammatory infiltrates.

Discussion

This study presents multiple lines of evidence in support of oncogenic properties of NF-κB in an epidermal cell line representing an early stage of squamous cell carcinoma development. Perhaps most importantly, overexpression of the NF-κB p65 subunit in immortalized HaCaT keratinocytes induced soft agar colony formation and tumorigenicity in immunodeficient mice; these are canonical criteria for full malignant transformation of solid tumor cells. In addition, the results presented here assign a role to TNF-α-induced NF-κB signaling in survival of HaCaT cells, particularly in the anchorage-independent state. This result is consistent with the earlier observation that mice deficient in TNF-α exhibit greatly reduced rates of carcinogen-induced skin tumor formation (20). Collectively, our results support the notion that deregulated NF-κB signaling, as observed in squamous cell carcinomas, supports several aspects of the malignant phenotype in this cell type.

However, our results seem to be in stark contrast to the well-characterized role of NF-κB as a tumor suppressor in normal murine and human keratinocytes. Notably, down-regulating NF-κB activity by targeted overexpression of the IκBSR in mouse epidermis is associated with epidermal hyperproliferation and increased rates of squamous carcinoma development (8) and NF-κB exerts growth inhibitory effects on normal mouse keratinocytes (10, 21). Similarly, abrogating NF-κB activity in human skin reconstructs xenotransplanted to mice leads to hyperproliferation and epidermal neoplasia (7). In light of this previous evidence, our data support the concept that immortalized epidermal keratinocytes at early stages of malignant transformation have accrued molecular alterations that enable a "switch" of NF-κB function from being a tumor suppressor to being a tumor promoter as recently proposed by Perkins (22) and Aggarwal (3).

Whereas tumor initiation usually involves inactivation of tumor suppressor genes, later stages of tumor development are frequently driven by oncogenes. It is presently unknown which initiating events have occurred in HaCaT keratinocytes that create permissive conditions for NF-κB to promote tumorigenicity of these cells. However, it is known that key tumor suppressors are altered in HaCaT cells. This includes increased telomerase activity (23), mutational inactivation of both alleles of p53 (24), silencing of p16INK4A expression by promoter methylation (25), and defective regulation of either p21 expression or function (26, 27). Consistent with earlier reports (23, 28), we found p21Waf1/Cip1

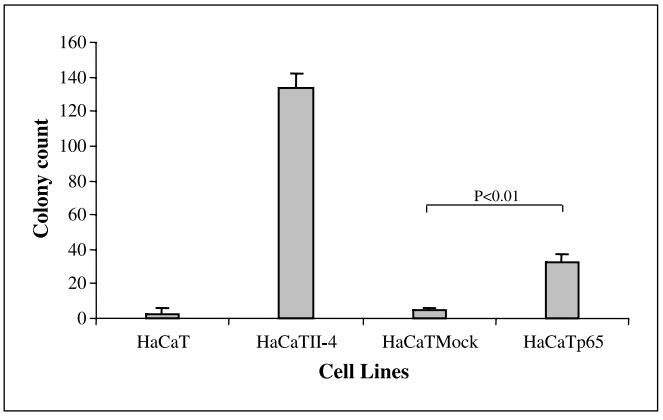

Figure 5. Overexpression of NF-κBp65 in HaCaT cells induces soft agar colony formation. Survival and growth of HaCaT, HaCaTII-4, HaCaTMock, and HaCaTp65 cells in soft agar was determined as described in Materials and Methods. Soft agar colonies of >50 cells were scored at 14 days in triplicate samples. *Columns,* mean of three independent experiments producing comparable results; *bars,* SD. $P < 0.01$, Student's *t* test.

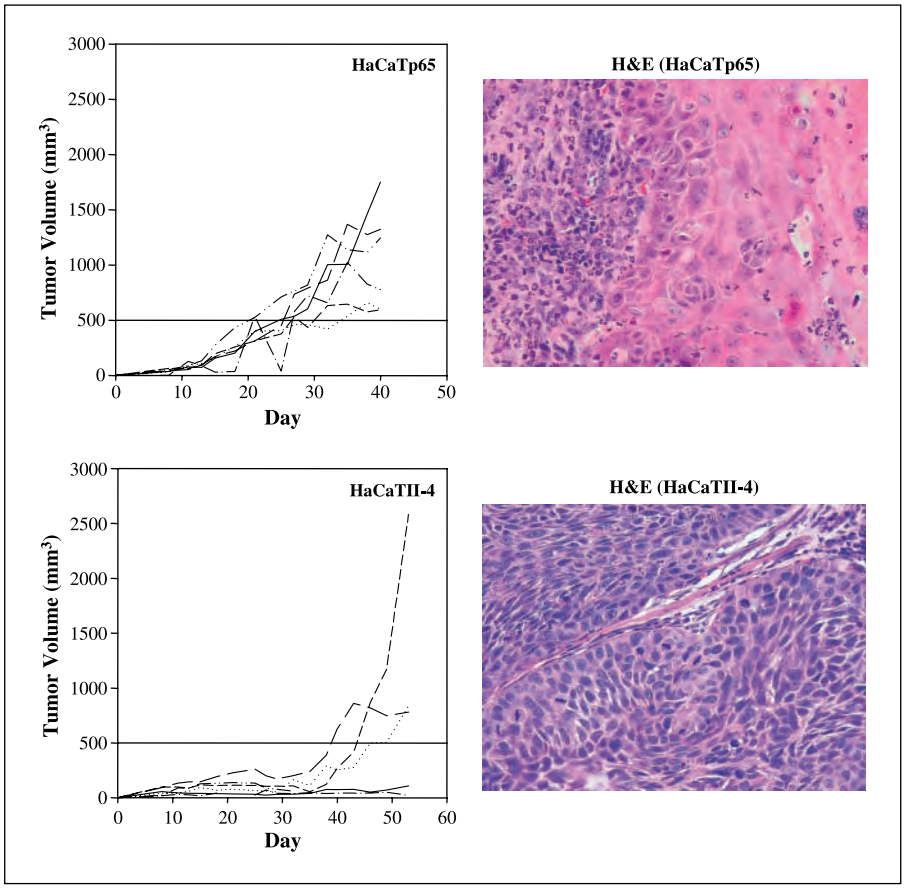

Figure 6. *In vivo* tumorigenicity of HaCaT keratinocytes overexpressing NF-κBp65. Single-cell suspensions of HaCaT cell variants were injected into the flanks of immunodeficient nude mice. HaCaTp65 and HaCaTII-4 tumor volumes were determined using calipers and calculated [(smallest diameter^2 × widest diameter) / 2] every 2 days for 2 month. Paraffin-embedded blocks containing HaCaTp65 and HaCaTII-4 tumors were processed for H&E staining.

protein not to be expressed in either HaCaT cells or HaCaTp65 cells under steady-state culture conditions.[4] Thus, as p21/Waf1/Cip1 is a target gene for NF-κB in normal keratinocytes, it may contribute to growth arrest on NF-κB activation in this cell type (29) but not in HaCaT cells (23). It remains to be determined whether loss of p21/Waf1/Cip1 expression, as observed in HaCaT cells, is sufficient for the manifestation of oncogenic properties of NF-κB activation. Alternatively, loss of either p53 or p16INK4A/ADP ribosylation factor (ARF) function in HaCaT cells may have unmasked transcriptional properties of NF-κB normally suppressed by p53 and/or ARF (22, 30). Additional studies are necessary to define the functional importance of NF-κB-dependent growth constraints in epidermal keratinocytes in preventing the emergence of malignant cells in the differentiating epidermis.

To our knowledge, this is the first report of tumorigenic conversion of HaCaT keratinocytes by overexpression of a single proto-oncogene. Previous work by the Fusenig group has shown that forced expression of oncogenic Ha-RasV12 was similarly capable of inducing tumorigenicity of HaCaT cells in experimental mice (19), a result confirmed in the present study using HaCaTII-4 cells. In contrast to HaCaTII-4 tumors, HaCaTp65 tumors revealed marked infiltration with inflammatory cells. Previous studies have implicated both tumor cell-autonomous effects of NF-κB signaling (31, 32) and "field effects" by chronic inflammation (32) in NF-κB driven epithelial tumor formation. However, in the case of HaCaTp65 tumors, the inflammatory infiltrate may, at least in part, be caused by excessive keratin deposition in HaCaTp65 tumors. Regardless of the relative contribution of the inflammatory response to tumorigenicity in mice, persistent overexpression of NF-κBp65 in HaCaT cells was clearly associated with tumor cell-autonomous roles of NF-κB activation, in support of epithelial cell survival and colony formation in soft agar. Thus, it seems likely that NF-κB signaling contributes in a tumor cell-autonomous fashion to the development and progression of hepatomas (31), colorectal carcinomas (32), and, as shown here, squamous cell carcinomas.

In summary, these results establish that deregulated NF-κB signaling exerts powerful oncogenic effects in an immortalized keratinocyte line. They lend support to the notion that NF-κB activity serves opposite roles in squamous cell carcinoma development, depending on tumor progression stage. Whereas NF-κB activation seems to curb transformation of normal uninitiated keratinocytes, it exacerbates the malignant phenotype of initiated, immortalized keratinocytes such as HaCaT cells.

Acknowledgments

Received 11/21/2005; revised 2/21/2006; accepted 3/9/2006.

Grant support: U.S. NIH grants CA106633 (A.P. Dicker) and CA81008 (U. Rodeck) and Department of Defense grant DAMD-17-02-1-0216 (U. Rodeck).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. N. Fusenig (Division of Carcinogenesis and Differentiation, German Cancer Research Center, Heidelberg, Germany) for providing HaCaT cells, and Yaping Sui, William R. Davidson, and Stephanie Lavorgna for technical assistance.

[4] M.R.D. Quadros and U. Rodeck, unpublished results.

References

1. Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-κB activity. Annu Rev Immun 2000;18:621–63.
2. Luque I, Gelinas C. Rel/NF-κB and IκB factors in oncogenesis. Semin Cancer Biol 1997;8:103–11.
3. Aggarwal BB. Nuclear factor-κB: the enemy within. Cancer Cell 2004;6:203–8.
4. Duffey DC, Chen Z, Dong G, et al. Expression of a dominant-negative mutant inhibitor-κBα of nuclear factor-κB in human head and neck squamous cell carcinoma inhibits survival, proinflammatory cytokine expression, and tumor growth *in vivo*. Cancer Res 1999;59:3468–74.
5. Loercher A, Lee TL, Ricker JL, et al. Nuclear factor-κB is an important modulator of the altered gene expression profile and malignant phenotype in squamous cell carcinoma. Cancer Res 2004;64:6511–23.
6. Aggarwal S, Takada Y, Singh S, Myers JN, Aggarwal BB. Inhibition of growth and survival of human head and neck squamous cell carcinoma cells by curcumin via modulation of nuclear factor-κB signaling. Int J Cancer 2004;111:679–92.
7. Dajee M, Lazarov M, Zhang JY, et al. NF-κB blockade and oncogenic Ras trigger invasive human epidermal neoplasia. Nature 2003;421:639–43.
8. van Hogerlinden M, Rozell BL, Ahrlund-Richter L, Toftgard R. Squamous cell carcinomas and increased apoptosis in skin with inhibited Rel/nuclear factor-κB signaling. Cancer Res 1999;59:3299–303.
9. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 1988;106:761–71.
10. Seitz CS, Deng H, Hinata K, Lin Q, Khavari PA. Nuclear factor κB subunits induce epithelial cell growth arrest. Cancer Res 2000;60:4085–92.
11. Seitz CS, Freiberg RA, Hinata K, Khavari PA. NF-κB determines localization and features of cell death in epidermis. J Clin Invest 2000;105:253–60.
12. Jost M, Huggett TM, Kari C, Boise LH, Rodeck U. Epidermal growth factor receptor-dependent control of keratinocyte survival and Bcl-xL expression through a MEK-dependent pathway. J Biol Chem 2001;276:6320–6.
13. Jost M, Huggett TM, Kari C, Rodeck U. Matrix-independent survival of human keratinocytes through an EGF receptor/MAPK-kinase-dependent pathway. Mol Biol Cell 2001;12:1519–27.
14. Jost M, Kari C, Rodeck U. An episomal vector for stable tetracycline-regulated gene expression. Nucleic Acids Res 1997;25:3131–4.
15. Stoker M, O'Neill C, Berryman S, Waxman V. Anchorage and growth regulation in normal and virus-transformed cells. Int J Cancer 1968;3:683–93.
16. Weaver VM, Lelievre S, Lakins JN, et al. β4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. Cancer Cell 2002;2:205–16.
17. Zahir N, Lakins JN, Russell A, et al. Autocrine laminin-5 ligates α6β4 integrin and activates RAC and NFκB to mediate anchorage-independent survival of mammary tumors. J Cell Biol 2003;163:1397–407.
18. Wang CY, Mayo MW, Baldwin AS, Jr. TNF-α and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. Science 1996;274:784–7.
19. Boukamp P, Stanbridge EJ, Foo DY, Cerutti PA, Fusenig NE. c-Ha-ras oncogene expression in immortalized human keratinocytes (HaCaT) alters growth potential *in vivo* but lacks correlation with malignancy. Cancer Res 1990;50:2840–7.
20. Moore RJ, Owens DM, Stamp G, et al. Mice deficient in tumor necrosis factor-α are resistant to skin carcinogenesis. Nat Med 1999;5:828–31.
21. Seitz CS, Lin Q, Deng H, Khavari PA. Alterations in NF-κB function in transgenic epithelial tissue demonstrate a growth inhibitory role for NF-κB. Proc Natl Acad Sci U S A 1998;95:2307–12.
22. Perkins ND. NF-κB: tumor promoter or suppressor? Trends Cell Biol 2004;14:64–9.
23. Kallassy M, Martel N, Damour O, Yamasaki H, Nakazawa H. Growth arrest of immortalized human keratinocytes and suppression of telomerase activity by p21WAF1 gene expression. Mol Carcinogenesis 1998;21:26–36.
24. Boukamp P, Peter W, Pascheberg U, et al. Step-wise progression in human skin carcinogenesis *in vitro* involves mutational inactivation of p53, rasH oncogene activation and additional chromosome loss. Oncogene 1995;11:961–9.
25. Chaturvedi V, Qin JZ, Denning MF, Choubey D, Diaz MO, Nickoloff BJ. Apoptosis in proliferating, senescent, and immortalized keratinocytes. J Biol Chem 1999;274:23358–67.
26. Chaturvedi V, Qin JZ, Denning MF, Choubey D, Diaz MO, Nickoloff BJ. Abnormal NF-κB signaling pathway with enhanced susceptibility to apoptosis in immortalized keratinocytes. J Dermatol Sci 2001;26:67–78.
27. Harvat BL, Jetten AM. Decreased growth inhibitory responses of squamous carcinoma cells to interferon-γ involve failure to recruit cki proteins into cdk2 complexes. J Invest Dermatol 2001;117:1274–81.
28. Facchinetti MM, De Siervi A, Toskos D, Senderowicz AM. UCN-01-induced cell cycle arrest requires the transcriptional induction of p21(waf1/cip1) by activation of mitogen-activated protein/extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase pathway. Cancer Res 2004;64:3629–37.
29. Basile JR, Eichten A, Zacny V, Munger K. 4 NF-κB-mediated induction of p21(Cip1/Waf1) by tumor necrosis factor α induces growth arrest and cytoprotection in normal human keratinocytes. Mol Cancer Res 2003;1:262–70.
30. Rocha S, Garrett MD, Campbell KJ, Schumm K, Perkins ND. Regulation of NF-κB and p53 through activation of ATR and Chk1 by the ARF tumour suppressor. EMBO J 2005;24:1157–69.
31. Pikarsky E, Porat RM, Stein I, et al. NF-κB functions as a tumour promoter in inflammation-associated cancer. Nature 2004;431:461–6.
32. Greten FR, Eckmann L, Greten TF, et al. Ikkβ links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell 2004;118:285–96.

3

Keratinocyte Culture in the Absence of Substrate Attachment

Monika Jost and Ulrich Rodeck

Summary

This chapter deals with experimental protocols and considerations related to the culture of epithelial cells under anchorage-independent conditions in liquid media. This technique has proven to be a powerful tool in studying the effects of loss of extracellular matrix interaction on crucial aspects of epithelial cell biology. Specifically, examining cells in the absence of substrate attachment, as described in this chapter, will allow the investigator to study the effect of growth factors independently of cell adhesion. Several methods are discussed relating to the preparation of tissue culture plates for suspension cultures and to the choice of a suitable cell culture medium.

Key Words:

Anchorage-independent growth; keratinocytes; adhesion; growth factors; differentiation.

1. Introduction

Adhesion of cells to extracellular matrix components is a crucial part of epithelial cell biology. Matrix attachment controls multiple aspects of cell physiology, including cytoarchitecture ***(1,2)***, cell cycle progression ***(3,4)***, and differentiation ***(5)***. In recent years, it has been realized that substrate attachment is also essential for the survival of epithelial cells, including keratinocytes ***(6–9)***. When removed from substrate, normal or immortalized epithelial cells will undergo apoptosis, a process termed "anoikis" ***(6)***. This aspect of substrate interaction may be rate-limiting to the spread of malignant cells during the metastatic process and has, thus, attracted intense interest. Furthermore, in multilayered epithelia such as the epidermis, detachment of cells from the basement membrane during the stratification process is part of the terminal differentiation program. The question of how terminal differentiation in the upper layers of stratifying epidermis relates to apoptosis incurred after losing contact with the basement membrane is yet to be resolved. However, the examination of molecular events induced by loss of matrix anchorage may help to distinguish matrix-dependent processes that contribute to differentiation and/or apoptosis.

The following brief protocols describe methods to culture either primary human keratinocytes isolated from neonatal foreskin or HaCaT cells, an immortalized keratinocyte cell line ***(10)*** in suspension culture. One of the key elements of these experiments is the medium composition, which is addressed separately. A crucial step in preventing the attachment of epithelial cells to tissue culture plates consists of covering plastic surfaces

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

with a substance that prevents cell attachment and the deposition of adhesion-competent extracellular matrix. This is commonly achieved by placing cells on either a poly–HEMA or agarose polymer. Both of these substrates prevent substrate attachment and extracellular matrix (ECM) deposition without interfering with cell-to-cell contact.

2. Materials

2.1. Media Preparation

1. MCDB153 medium (Sigma-Aldrich; St. Louis, MO; cat. no. M7403).
2. Amino acids for media preparation (L-isomers from Sigma-Aldrich [cell culture grade]): histidine–HCl (cat. no. H9511); isoleucine (cat. no. I7383); methionine (cat. no. M2893); phenylalanine (cat. no. P5030); tryptophan (cat. no. T0271); and tyrosine (cat. no. T1145). Amino acids are stored at room temperature, except for tyrosine (stored at 4°C).
3. Sodium bicarbonate (Sigma-Aldrich; cat. no. S5761).
4. Ethanolamine (Sigma-Aldrich; cat. no. E9508).
5. *O*-Phosphoethanolamine (Sigma-Aldrich; cat. no. 0503).
6. Hydrocortisone (Sigma-Aldrich; cat. no. H0888).
7. Bovine serum albumin fraction V, heat shock, fatty acid free (Roche Diagnostics Corporation-Roche Applied Science; Indianapolis, IN; cat. no. 0100062).

2.2. Suspension Culture

1. SeaKem LE agarose (Cambrex; Rockland, ME; cat. no. 50001).
2. Poly(2-hydroxyethyl methacrylate (Poly–HEMA); (Sigma-Aldrich; cat. no. P3932).

3. Methods

3.1. Cell Culture Medium for Anchorage-Independent Growth

The choice of cell culture medium is crucial for the interpretation of phenomena observed in liquid suspension culture. In the following, we will discuss several examples that illustrate how the composition of culture media can affect experimental outcomes. Although it is impossible to determine a "gold standard" medium for experiments in liquid suspension culture, the following considerations may serve as starting point for designing media composition.

3.1.1. Cell Culture Medium for Investigating the Effect of Growth Factors and Cytokines on Anchorage-Independent Growth and/or Apoptosis

To examine the effect of growth factors and cytokines on anchorage-independent cell cycle progression and/or apoptosis, a chemically defined base medium is essential. Preparation of a suitable base medium, in this chapter referred to as MCDB153 base medium ***(11)***, is described in **Subheading 3.1.3.** MCDB153 base medium is buffered with carbonate and contains essential amino acids, hydrocortisone, ethanolamine, and *o*-phosphoethanolamine. This medium will sustain viability albeit not cell cycle progression of keratinocytes for 48–72 h in the attached state. Growth factors and cytokines of interest may be added as required by the experimental design. It is recommended to add fatty-acid free bovine serum albumin as a carrier protein to avoid loss of cytokines as a result of nonspecific binding to the walls of the culture vessel. Any undefined components used for routine culture, including bovine pituitary extract and fetal bovine serum, should be omitted. Commercially available keratinocyte

media similar to MCDB153 base medium may be used as an alternative, as long as they do not contain proprietary or undisclosed components, designed to ensure optimal growth and survival of keratinocytes in adherent culture.

3.1.2. Cell Culture Medium for Investigating Mechanisms of Differentiation and Cell–Cell Adhesion

The MCDB153 base medium is distinguished by a comparatively low Ca^{2+} concentration (29 μ*M*), designed to prevent Ca^{2+}-induced differentiation of keratinocytes under routine culture conditions. An important corollary of low ambient Ca^{2+} in suspension culture is prevention of the formation of cell clumps or spheroids that do form if the Ca^{2+} concentration is higher than 300 μ*M*. This is likely a result of the fact that Ca^{2+} contributes to cadherin-based cell–cell adhesion ***(12,13)***. In effect, the use of low Ca^{2+} media ensures that normal keratinocytes will remain as single cells or loose aggregates during suspension culture; such aggregates can be dispersed by gentle pipetting at least during the first 4 d of suspension culture. Thus, not only cell–matrix but also cell–cell adhesion-mediated signaling events will be prevented in low Ca^{2+} cell culture medium. If cell–cell adhesion is desired, it can be restored by supplementing the base medium with Ca^{2+} at concentrations greater than 500 μ*M*. It should be noted that commercially available media for keratinocyte culture contain Ca^{2+} at a range of concentrations. For example, KGM from GIBCO-Invitrogen (Carlsbad, CA) contains 100 μ*M* of Ca^{2+}, whereas MCDB153 base medium contains only 29 μ*M* of Ca^{2+}. We observed that KGM (GIBCO-Invitrogen) allows the differentiation of keratinocytes in suspension to proceed to a greater extent than MCDB153 base medium as described here (results unpublished). These differences highlight the importance of considering Ca^{2+} concentration as it relates to cellular phenotypes in suspension culture (*see* **Note 1**).

3.1.3. Preparation of MCDB153 Base Medium

1. To 900 mL of dH_2O, add MCDB153 base medium powder (1 bottle for 1 L of final volume) and stir. Rinse the bottle with a little more dH_2O and add to mixture. Stir to dissolve.
2. Add 1.176 g of sodium bicarbonate (Sigma; cat. no. S5761).
3. Ensure that pH is 6.85.
4. Add amino acids as follows:

Amino acid	Final concentration	MW	Add per 1 L of medium
Histidine	$2.4 \times 10e^{-4}$	209.6	50 mg
Isoleucine	$7.5 \times 10e^{-4}$	131.2	98 mg
Methionine	$9.0 \times 10e^{-5}$	149.2	13 mg
Phenyalanine	$9.0 \times 10e^{-5}$	165.2	15 mg
Tryptophan	$4.5 \times 10e^{-5}$	204.2	9 mg
Tyrosine	$7.5 \times 10e^{-5}$	225.2	17 mg

All amino acids are stored at room temperature, except for tyrosine (stored at 4°C).

5. Stir to dissolve (approx 60 min). Adjust volume to 1 L.
6. Adjust pH to 7.6 with 50% w/v NaOH.
7. Filter through 0.2-μm filter; make aliquots of 500 mL and store at –20°C.
8. To 250 mL MCDB-Base medium, add the following:

	Final concentration
250 μL ethanolamine (0.1 *M*)	0.1 m*M*
250 μL *O*-phosphoethanolamine (0.1 *M*)	0.1 m*M*
25 μL hydrocortisone (5 m*M*)	0.5 μ*M*

3.2. Preparation of Culture Plates for Nonadherent Cell Culture

3.2.1. Protocol A: Preparation of Poly–HEMA–Coated Plates

3.2.1.1. Materials

1. 95% Ethanol.
2. Tissue culture medium (*see* **Subheading 2.1.**).
3. Tissue culture-treated plates with various surface area configurations (six-well plates are useful for many applications).
4. Phosphate-buffered saline without Ca^{2+} and Mg^{2+} (sterile).

3.2.1.2. Method

1. Prepare a stock solution of Poly–HEMA by dissolving 10 mg/mL in 95% ethanol while stirring vigorously. To enhance solubility, incubate the solutions in a tightly capped bottle overnight at 37°C. Remove any undissolved material by centrifugation at about 500*g* for 30 min at room temperature (*see* **Note 2**).
2. Coat plates with the solution in a biosafety cabinet. Use approx 0.12 mL/cm^2 surface area (i.e., 1 mL/well of a six-well plate) and let the ethanol evaporate by leaving the plates open in the cabinet.
3. Repeat this process twice to cover the surface completely.
4. Wash the plates several times with phosphate-buffered saline (about 0.25 mL/cm^2). The plates are then ready for use. Unused plates can be sealed with plastic wrap and stored at 4°C.

3.2.2. Protocol B: Preparation of Agarose-Coated Plates

3.2.2.1. Materials

1. Agarose (molecular biology grade).
2. Tissue culture medium (*see* **Subheading 3.1.**).
3. Tissue culture-treated plates of suitable sizes (e.g., six-well plates).
4. Fatty-acid-free bovine serum albumin (optional, *see* **Subheading 3.1.**).

3.2.2.2. Preparation of Agarose-Coated Plates

1. Prepare a 0.9% (w/v) agarose stock solution by weighing 0.9 g agarose into 100 mL of medium (growth-factor free medium, *see* **Subheading 3.2.2.3.**) (*see* **Note 3**).
2. Boil the agarose suspension in a microwave oven to dissolve the agarose and sterilize the solution; alternatively, the solution can be autoclaved in the liquid cycle and should be used before it solidifies.
3. Pipet the solution into plates in a biosafety cabinet. Add approx 0.24 mL per cm^2 surface area (2 mL/well of a six-well plate).
4. Leave the plates open in the biosafety cabinet at room temperature until the agarose has solidified.
5. The plates are ready for use. For storage, add a small amount of medium to each well (about 0.2 mL/cm^2) to prevent drying out, seal with plastic wrap and store at 4°C.

3.2.3. Suspension Culture and Cell Harvest

1. Trypsinize cells and inactivate trypsin using established procedures (either with serum or soybean trypsin inhibitor).
2. Count cells by Trypan blue exclusion assay and resuspend in medium (depending on experimental conditions; *see* **Subheading 3.1.3.**). A recommended starting cell density is $3–5 \times 10^5$ viable cells per milliliter (HaCaT or normal keratinocytes). Use at least 2 mL of medium per well (six-well plate) or more, depending on the number of cells required for

subsequent analysis. When using MCDB153 base medium (*see* above), add 0.2 % (w/v) fatty-acid-free bovine serum albumin.
3. Maintain cells in suspension for desired time intervals up to 72 h. If longer incubation times are required, culture media need to be replaced by centrifugation of the cells, resuspension in fresh medium, and reseeding on agarose.
4. When removing aliquots of cells for analysis, mix the culture by gentle pipetting with a serological pipet, then remove the desired volume and analyze cells.
5. For clonogenic assays, remove 50 to 100 µL cell suspension (~1.5–5 × 10^4 cells) and seed into 24-well plates in optimal growth medium containing all supplements.
6. For molecular or biochemical analyses (e.g., protein or ribonucleic acid extraction), harvest a sufficient number of cells by centrifugation at 400*g* and process as required.

4. Notes

1. HaCaT cells should be cultured for several days in keratinocyte medium before assay. Serum-containing media that are frequently used for routine maintenance of HaCaT cells may exert carryover effects that can affect viability or differentiation in suspension culture, thus making it difficult to distinguish effects of single growth factors
2. It is necessary to repeat coating with Poly–HEMA at least twice to completely cover the surface area. Uncovered areas will allow partial attachment of cells. This can pose a potential source of error if cell numbers are to be determined.
3. It is best to use agarose-coated plates shortly after preparation; longer storage (i.e., for several days or longer) can lead to shrinkage or cracking of the agarose. When this occurs, some cells will manage to slip underneath the agarose while in suspension and grow attached on the plastic, which, as mentioned above, is a potential source of error. For repeated use, the agarose stock solution can be stored at 4°C once it has solidified and may be reheated in a microwave oven whenever more plates are needed. To equilibrate the agarose, a medium similar to the one used in the experiment should be used, with respect to Ca^{2+} concentration. It is not recommended to use growth factor-supplemented medium for the preparation of the agarose solution, since proteins will be denatured by heat (either in the autoclave or in the microwave). Although this is not assumed to negatively affect the experiment, it will waste expensive growth supplements.

References

1 Wang, N., Naruse, K., Stamenovic, D., Fredberg, J. J., Mijailovich, S. M., Tolic-Norrelykke, I. M., et al. (2001) Mechanical behavior in living cells consistent with the tensegrity model. *Proc. Natl. Acad. Sci. USA* **98,** 7765–7770.
2 Ingber, D. E. (2003) Tensegrity II: how structural networks influence cellular information processing networks. *J. Cell Sci.* **116,** 1397–1408.
3 Bottazzi, M. E., Zhu, X., Bohmer, R. M., and Assoian, R. K. (1999) Regulation of p21(cip1) expression by growth factors and the extracellular matrix reveals a role for transient ERK activity in G1 phase. *J. Cell Biol.* **146,** 1255–1264.
4 Roovers, K., Davey, G., Zhu, X., Bottazzi, M. E., and Assoian, R. K. (1999) Alpha5beta1 integrin controls cyclin D1 expression by sustaining mitogen-activated protein kinase activity in growth factor-treated cells. *Mol. Biol. Cell* **10,** 3197–3204.
5 Jones, J., Sugiyama, M., Speight, P. M., and Watt, F. M. (1996) Restoration of alpha v beta 5 integrin expression in neoplastic keratinocytes results in increased capacity for terminal differentiation and suppression of anchorage-independent growth. *Oncogene* **12,** 119–126.
6 Frisch, S. M. and Francis, H. (1994) Disruption of epithelial cell-matrix interactions induces apoptosis. *J. Cell Biol.* **124,** 619–626.

7 Boudreau, N., Werb, Z., and Bissell, M. J. (1996) Suppression of apoptosis by basement membrane requires three-dimensional tissue organization and withdrawal from the cell cycle. *Proc. Natl. Acad. Sci. USA* **93,** 3509–3513.
8 Rodeck, U., Jost, M., DuHadaway, J., Kari, C., Jensen, P. J., Risse, B., and Ewert, D. L. (1997) Regulation of Bcl-xL expression in human keratinocytes by cell-substratum adhesion and the epidermal growth factor receptor. *Proc. Natl. Acad. Sci. USA* **94,** 5067–5072.
9 Frisch, S. M. and Screaton, R. A. (2001) Anoikis mechanisms. *Curr. Opin. Cell Biol.* **13,** 555–562.
10 Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A., and Fusenig, N. E. (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* **106,** 761–771.
11 McNeill, H. and Jensen, P. J. (1990) A high-affinity receptor for urokinase plasminogen activator on human keratinocytes: characterization and potential modulation during migration. *Cell Regul.* **1,** 843–852.
12 Nagafuchi, A., Shirayoshi, Y., Okazaki, K., Yasuda, K., and Takeichi, M. (1987) Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. *Nature* **329,** 341–343.
13. Wheelock, M. J. and Jensen, P. J. (1992) Regulation of keratinocyte intercellular junction organization and epidermal morphogenesis by E-cadherin. *J. Cell Biol.* **117,** 415–425.

[CANCER RESEARCH 64, 3934–3939, June 1, 2004]

Complex Regulation of Signal Transducers and Activators of Transcription 3 Activation in Normal and Malignant Keratinocytes

Marlene R. D. Quadros,[1] Francesca Peruzzi,[2] Csaba Kari,[1] and Ulrich Rodeck[1]

[1]Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, and [2]Center for Neurovirology and Cancer Biology, Temple University, Philadelphia, Pennsylvania

ABSTRACT

Previous work implicated activation of the signal transducer and activator of transcription (STAT)3 downstream of the epidermal growth factor receptor (EGFR) in the malignant phenotype of squamous carcinoma cells (SCC). Here, we show that EGFR-dependent STAT3 activation is restricted to malignant keratinocytes. Specifically, constitutive and epidermal growth factor-induced phosphorylation of STAT3 on Y705 was observed only in SCC but not in either immortalized (HaCaT) or normal keratinocyte strains. Furthermore, STAT3 activation as determined by DNA binding assays was restricted to SCC and dependent on EGFR activation. Forced expression of EGFR in immortalized keratinocytes (HaCaT cells) was associated with enhanced EGFR activation but not STAT3-Y705 phosphorylation. EGFR-dependent activation of mitogen-activated protein kinase (MAPK) kinase 1 negatively regulated STAT3-Y705 phosphorylation in normal and malignant keratinocytes. Together, these results underscore that EGFR activation is required but not sufficient for STAT3 activation to occur in malignant keratinocytes. They also highlight complex regulation of STAT3 phosphorylation through EGFR activation including negative regulation via the MAPK kinase/MAPK signaling pathway.

INTRODUCTION

Activation of the epidermal growth factor receptor (EGFR) contributes to multiple aspects of epithelial cell biology relevant to malignant transformation. These include proliferation, migration, and invasion, and modulation of differentiation. Only recently has it become apparent that the EGFR also contributes to survival of epithelial cells including normal and malignant human keratinocytes (1–3). If the EGFR is blocked, normal keratinocytes are significantly more susceptible to induction of apoptotic death by cellular stressors including UVB radiation (4) and matrix detachment (1, 5). Other members of the EGFR family, notably erbB2, serve similar functions relevant to epithelial cell survival (6–10). We have identified one intracellular target of EGFR activation that supports keratinocyte survival (11, 12). Specifically, EGFR activation through endogenous and exogenous ligands is associated with up-regulation of Bcl-x_L, an antiapoptotic member of the Bcl-2 protein family. By contrast, expression of the proapoptotic Bcl-2 family members Bad, Bak, and Bax is not affected by EGFR activation. Thus, EGFR blockade is associated with a proapoptotic bias in the balance of pro- and antiapoptotic Bcl-2 family members. Similar results have since been reported by several other groups (2, 13, 14).

One objective of our previous work was to understand which signaling pathway(s) lead from the EGFR to enhanced cell survival through higher levels of Bcl- x_L expression. These studies demonstrated that EGFR-dependent signals relevant to keratinocyte survival and Bcl- x_L expression are channeled in part through the mitogen-activated protein kinase (MAPK) kinase (MEK)/MAPK pathway (15). EGFR-dependent MEK/MAPK activation additionally enhances epithelial cell survival by phosphorylation and functional inactivation of the proapoptotic Bcl-2 family member Bad (16). In addition to MEK/MAPK, activation of the signal transducer and activator of transcription (STAT)3 has been implicated in epithelial cell survival and Bcl-x_L expression. STAT3 supports oncogenic transformation of mouse fibroblasts (17, 18) and survival of human squamous carcinoma cells (SCC), at least in part through up-regulation of Bcl-x_L (19). Conversely, EGFR blockade down-regulates STAT3 phosphorylation and DNA binding in some SCC lines consistent with EGFR-dependent regulation of STAT3 activity in this cell system. However, in immortalized keratinocytes, EGFR activation had no apparent effect on STAT3 phosphorylation and, moreover, inhibition of STAT activity by inducible expression of dominant-negative STAT3 constructs had no effect on Bcl- x_L expression in these cells (5).

Collectively, these previous observations raised the issue of whether EGFR-dependent STAT3 activation and its sequelae were restricted to malignant keratinocytes. The present study addresses this question by using normal human keratinocyte cultures, an immortalized keratinocyte line (HaCaT), and a panel of SCC lines exhibiting different levels of EGFR expression and activation states. We demonstrate that: (*a*) STAT3 phosphorylation on Y705 is a tumor-associated phenomenon in squamous carcinoma cells *in vitro*; (*b*) STAT3 phosphorylation on Y705 occurs through EGFR-dependent and -independent pathways in malignant cells; (*c*) overexpression of the EGFR is not sufficient for STAT3 phosphorylation in immortalized keratinocytes (HaCaT); and (*d*) DNA binding of activated STAT3 is restricted to malignant tumor cells and strictly dependent on EGFR activation. We conclude that STAT3 activation in normal and malignant keratinocytes is a tumor-associated phenomenon linked to deregulated EGFR signaling.

MATERIALS AND METHODS

Reagents and Cells. Properties of the EGFR antagonistic mAb425 have been described earlier (20, 21). Inhibitors to MEK1 (U0126), phosphatidylinositol 3′-kinase (LY294002), SRC-kinases (PP1 and PP2), and the tyrphostins AG490 and AG1478 were purchased from Calbiochem-Novabiochem (San Diego, CA). Rabbit polyclonal antibodies to EGFR were from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling/NEB (Beverly, MA), and to β-actin from Amersham Biosciences (Piscataway, NJ). Antibodies to signal transduction components (STAT3, phospho-STAT3, p42/44 MAPK, and phospho-p42/44 MAPK) were from Cell Signaling Technology or Santa Cruz Biotechnology. Hemagglutinin tag antibodies were from Covance (Richmond, CA). Antiphosphotyrosine antibody PY20 was from Transduction Laboratories (San Diego, CA).

Normal foreskin keratinocytes were initiated and maintained in culture as described earlier (22). Head and neck carcinoma cell lines SCC 9 and SCC 12 derived from facial skin were a kind gift from Dr. Jim G. Rheinwald. Head and neck carcinoma cell lines FaDu and Det562 were from the American Type Culture Collection (Rockville, MD). A431 cells were derived from a vulval SCC and originally provided by Dr. Ira Pastan (National Cancer Institute, NIH, Bethesda, MD). Immortalized keratinocytes (HaCaT cells) were from Dr. Norbert Fusenig (23). HaCaT, SCC 9, SCC 12, FaDu, A431, and Det562 cells were maintained in W489 medium (24) supplemented with 2% FCS. For

Received 1/22/04; revised 3/15/04; accepted 3/23/04.

Grant support: NIH Grant (CA-81008) and United States Department of the Army (DAMD17–02-1–0216; U. Rodeck).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Ulrich Rodeck, Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, 233 South 10th Street, BLSB 319, Philadelphia, PA 19107. Phone/Fax: 215/503-5622; E-mail: Ulrich.rodeck@mail.tju.edu.

experiments, all of the cell lines were seeded at subconfluency in either complete MCDB medium (12) for primary keratinocyte cultures or W489 supplemented with 2% FCS for HaCaT cells and SCC lines. After attachment, medium was replaced with MCDB base medium (12) supplemented with growth factors and inhibitors of signal transduction components as indicated. Inhibitors were diluted from DMSO stocks directly into the culture medium. The DMSO concentration was adjusted to <0.5% in all of the conditions including controls. After 48 h cells were harvested for analysis.

cDNA Constructs and Transfections. A plasmid containing dominant-negative MEK1 (MKK1−8E) was cloned into pCEPTetP and transfected into HaCaT cells expressing tTA as described previously (15). This system allows transgene induction by removal of tetracycline from culture medium (25). Similarly, dominant-negative STAT3D and STAT3F were cloned into pCEPTetP and transfected into HaCaT-tTA1 cells as described previously (15). The full-length EGFR cDNA was cloned into the pIRESpuro2 vector (Clontech, Palo Alto, CA) followed by stable transfection into HaCaT cells. Transfections were performed using Fugene 6 (Roche Molecular Biochemicals, Indianapolis, IN). Briefly, cells were seeded at a density of $\sim 5 \times 10^3/cm^2$ in W 489 medium supplemented with 2% FCS and allowed to attach overnight. The next day transfections were performed using 0.5 μg DNA and 1.7–2.0 μl Fugene 6 per 10^5 cells following the manufacturer's protocol. Selection was started 48–72 h after transfection in W489 medium supplemented with 2% FCS and puromycin at 1 μg/ml (Sigma-Aldrich, St. Louis, MO).

Immunoprecipitation and Immunoblot Analyses. Samples for immunoprecipitation were collected by washing adherent cells once in ice-cold PBS followed by scraping into cold lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.0 mM EGTA, 1.0 mM EDTA, 10% glycerol, 1.0% Triton-X-100, 50 mM NaF, 10 mM Na4P2O7 containing freshly added protease inhibitors (Complete protease inhibitor mixture; Roche Molecular Biochemicals), 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na3VO4]. After lysis for 20 min on ice, cell extracts were centrifuged at 15,000 × *g* and supernatants stored at 70°C until further use. Protein content was determined using the BCA method (Pierce Chemical Co., Rockford, IL). Equal amounts of protein were precleared with 20 μl Protein A/G Plus agarose (Santa Cruz Biotechnology) for 1 h at 4°C. Precleared lysates were then mixed with 2.0 μg of primary rabbit EGFR antibody (Santa Cruz Biotechnology) and 20 μl protein A/G Plus agarose and incubated overnight at 4°C. Precipitates were washed four times with lysis buffer, solubilized in 1× reducing Laemmli buffer [62.5 mM Tris-Cl (pH 6.8), 1% SDS, 10% glycerol, 0.5 M 2-mercaptoethanol, or 0.1 M dithiothreitol], boiled for 3–5 min, and then subjected to SDS-PAGE followed by Western blot as described below.

Samples for Western blots were collected by washing cells once in cold PBS and lysis in Laemmli buffer followed by boiling for 3–5 min. Equal amounts of protein were separated by SDS-PAGE under reducing conditions and blotted onto nitrocellulose membranes (Millipore). Membranes were blocked using 5% dry milk in PBS or 5% dry milk, 0.05% Tween 20 (Sigma Aldrich) in Tris-buffered saline, and then incubated with primary antibodies in PBS or 5% BSA, 0.05% Tween 20 in Tris-buffered saline, followed by incubation in dilutions of horseradish peroxidase-conjugated secondary antibodies in the same buffers. After antibody incubations, blot membranes were washed in 0.5% Tween 20 in Tris-buffered saline. Signals were visualized by chemiluminescence using reagents from Pierce Chemical Co. according to the manufacturer's instructions. After detection, blots were washed and stripped using Restore Western Blot Stripping Buffer (Pierce Chemical Co.) and used for additional antibody incubations.

Electrophoretic Mobility Shift Assays. Cells were starved for 14 h in growth factor-free medium and then stimulated with epidermal growth factor (EGF) for 15 min. Whole cell lysates were prepared from control and EGF-treated cells. Briefly, after washing twice with PBS, cells were harvested in 1 ml of PBS and recovered by centrifugation. Cells were resuspended in twice the pellet volume using high salt buffer [20 mm HEPES (pH 7.9), 20 mm NaF, 1 mm Na_3VO_4, 1 mm EGTA, 1 mm EDTA, 1 mm DTT, 400 mm NaCl, 20% glycerol, 0.1% Triton X-100, 1 mm phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin] and were kept for 30 min at 4°C under vigorous agitation. The lysates were centrifuged at 15,000 × *g* for 20 min at 4°C, and protein concentrations of the clarified lysates were determined by Bradford assay. DNA binding was performed by adding 15–20 μg of protein lysates to 18 μl of reaction mixture containing 65 mm NaCl, 10 mm HEPES (pH 7.9), 1 mm DTT, 2% Ficoll 400, 4% glycerol, and 1 μg of poly(deoxyinosinic-deoxycytidylic acid; Amersham Life Science Inc.) The mixture was preincubated on ice for 15 min followed by addition of 30,000 cpm (1–1.5 ng) of the double-stranded labeled oligonucleotide (high-affinity SIE sequence; Ref. 26) and additionally incubated for 30 min at room temperature. Samples were analyzed in a 20 × 20 cm 5% polyacrylamide gel with a bisacrylamide:acrylamide ratio of 1:39 containing 2.5% glycerol and 0.5 × TBE (45 mm Tris base and 1 mm EDTA). Electrophoresis was carried out at 175 V until the faster migrating bromphenol blue dye was ~4 cm from the bottom edge. The gel was dried and subjected to autoradiography using KodakXAR5 film for 3 days with intensifying screens at −70°C.

RESULTS

Expression and Ligand-Induced Phosphorylation of the EGFR in Normal and Immortalized Keratinocytes and in SCCs. In an earlier study we observed that EGFR activation contributes to STAT3 phosphorylation on Y705 in A431 SCCs but not in immortalized HaCaT keratinocytes (5). These two cell lines were derived from malignant and normal epidermis, respectively. In addition, these cell lines are distinguished by high (A431) and low levels (HaCaT) of EGFR expression. On the basis of these results, we hypothesized that EGFR-dependent STAT3 phosphorylation is restricted to epithelial cells with deregulated EGFR expression and signaling and, hence, a tumor-associated phenomenon. To test this hypothesis we screened a panel of 4 normal primary keratinocytes and 5 malignant SCCs derived either from skin (SCC9, SCC12, and A431) or oral mucosa (all others) for EGFR expression and activation states. For control purposes we included immortalized HaCaT keratinocytes derived from skin (23). We observed that all of the cell lines tested expressed the EGFR albeit at different levels (Fig. 1). When compared with four primary keratinocyte strains and to immortalized HaCaT keratinocytes, the malignant cell lines (Det562, FaDu, SCC12, SCC9, and A431) expressed higher levels of the EGFR with A431 cells expressing the highest. Next, we tested the EGFR phosphorylation state in these cell lines in the presence and absence of exogenous EGF in chemically defined, serum-free media (22). Short-term exposure to 10 ng/ml EGF for 15 min induced EGFR phosphorylation in all of the cell lines as determined by immunoprecipitation of the EGFR followed Western blot analysis using a phosphotyrosine-specific antibody (Fig. 1). The strength of the signal varied with higher levels of EGFR phosphorylation in the transformed cells and the highest level

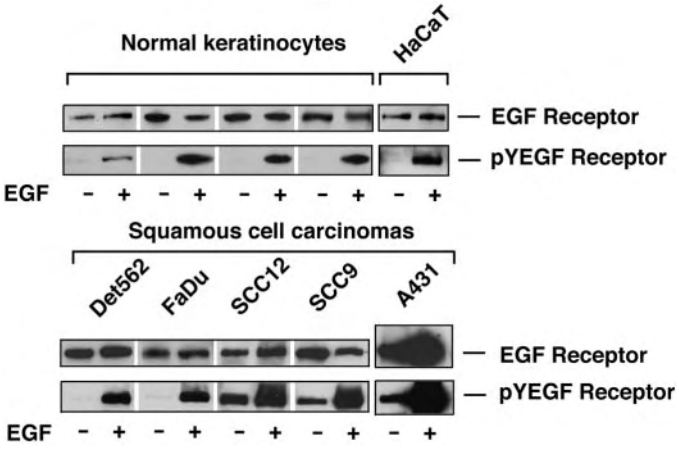

Fig. 1. Expression and phosphorylation of the epidermal growth factor receptor (EGFR) in normal and transformed keratinocytes. Cell extracts were prepared from the cell lines as indicated after stimulation of growth factor-starved cultures with epidermal growth factor (*EGF;* 10 ng/ml) for 15 min. EGFR expression and phosphorylation levels were determined using antibodies to the extracellular domain of the EGFR and PY20, respectively, by immunoprecipitation and Western blot analyses. For loading controls please refer to Fig. 2, which shows expression levels of STAT3 in the same samples.

in A431 cells. In the absence of exogenous EGF neither the normal keratinocyte strains nor HaCaT cells contained phosphorylated EGFR at detectable levels. In contrast, 3 of 5 malignant cell lines (SCC9, SCC12, and A431) revealed "constitutive" EGFR phosphorylation in the absence of exogenous EGF. In conclusion, the panel of cell lines assembled represents a diverse array of EGFR expression and activation states consistent with deregulated EGFR activation in the malignant cells.

Expression and EGFR-Dependent STAT3 Phosphorylation in Normal and Malignant Keratinocytes. Next, we determined expression and phosphorylation of STAT3 in the panel of cell lines under investigation (Fig. 2). As determined by Western blot analysis all of the cell lines tested expressed STAT3. Consistent with our earlier results (15) addition of EGF to the culture medium induced robust phosphorylation of STAT3 on Y705 in A431 but not in HaCaT cells. STAT3 tyrosine phosphorylation was not detected in any of the 4 normal keratinocyte strains tested either in the presence or absence of exogenous EGF. By contrast, all of the carcinoma cell lines except Det562 revealed STAT3 Y705 phosphorylation in the presence of exogenous EGF. Interestingly, SCC9, SCC12, and FaDu cell extracts contained phosphorylated STAT3-Y705 also in the absence of exogenous EGF. To assess whether the EGFR was functional in the cell lines under investigation we also determined MAPK phosphorylation in all of the conditions. We had observed previously that MAPK phosphorylation in normal keratinocytes and HaCaT cells strictly correlates with EGFR activation under the experimental conditions chosen (15). Consistent with these earlier results, all of the cell lines under investigation here responded to EGF treatment with robust MAPK phosphorylation. The comparatively strong phospho-MAPK signal in some of the keratinocyte extracts is likely due to higher protein concentrations in these samples as evidenced by equally higher levels of STAT3 protein in the same samples.

These results raised the issue of whether STAT3 tyrosine phosphorylation seen in the absence of exogenous EGF in SCC lines was due to EGFR activation via autocrine EGFR ligands. To probe the contribution of endogenous EGFR ligands to STAT3 phosphorylation in FaDu, SCC9, SCC12, and A431 cells we used the EGFR antagonistic tyrphostin AG1478 (Fig. 3*A*). AG1478 abrogated EGFR phosphorylation (data not shown) and markedly inhibited EGFR-dependent

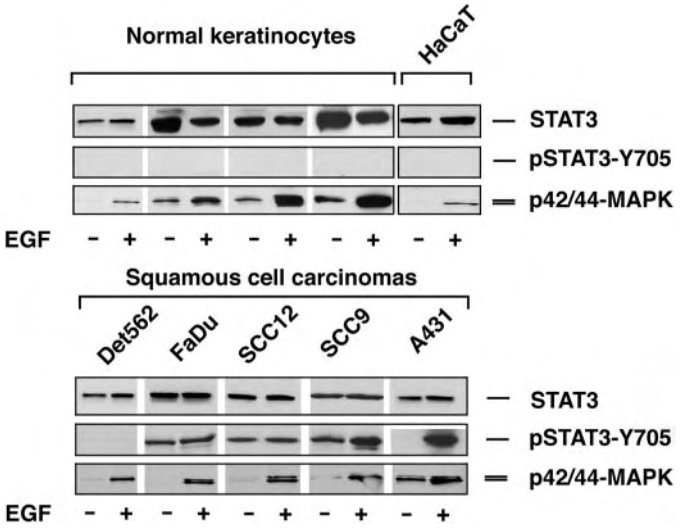

Fig. 2. Expression and phosphorylation states of signal transducer and activator of transcription (*STAT*)3 in normal and transformed keratinocytes. Cell extracts were prepared as described in the legend to Fig. 1 and immunoblots probed using antibodies to STAT3, STAT3 phosphorylated on Y705, and phosphorylated mitogen-activated protein kinase (*MAPK*) as indicated.

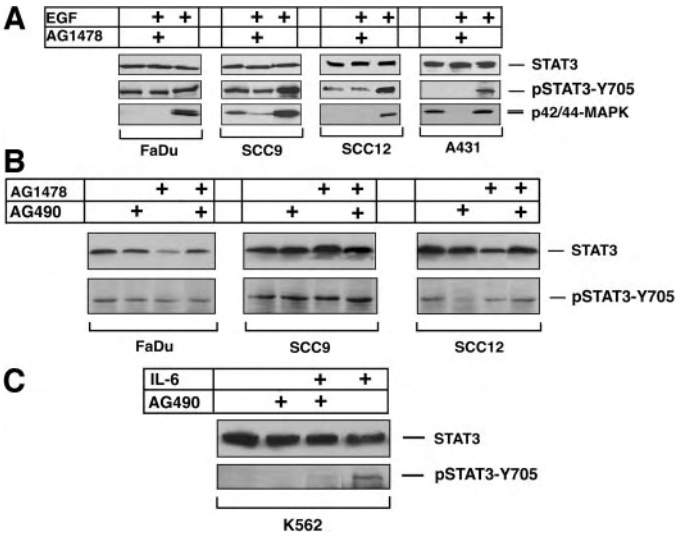

Fig. 3. Effects of inhibitors of the epidermal growth factor (*EGF*) receptor (*AG1478*) and of Janus-activated kinase (*AG490*) alone and in combination on signal transducer and activator of transcription (*STAT*)3 tyrosine phosphorylation in squamous carcinoma cells. Growth factor-starved cells were pretreated with inhibitors for 1 h as indicated and then stimulated with EGF (10 ng/ml) for 15 min before preparing cell extracts. *A* demonstrates that EGF receptor inhibition by treatment with AG1478 (10 μM) did not affect STAT3 tyrosine phosphorylation in FaDu, SCC9, and SCC12 in the absence of exogenous EGF. *B* shows effects of the Janus-activated kinase inhibitor AG490 at 100 μM on STAT3-Y705 phosphorylation in the absence of exogenous EGF in these cell lines. *C* documents that AG490 (100 μM) inhibited interleukin (*IL*) 6-dependent STAT3-Y705 phosphorylation in K562 cells.

MAPK phosphorylation in all 4 of the SCC lines tested. Yet, AG1478 treatment had only marginal effects on STAT3 phosphorylation in those 3 cell lines that demonstrated STAT3-Y705 phosphorylation in the absence of exogenous EGF. Notably, however, AG1478 inhibited the effect of exogenous EGF on STAT3-Y705 phosphorylation in SCC9 and SCC12 cells, and abrogated STAT3 phosphorylation altogether in A431 cells. To ascertain a potential role of gp130 activation in EGFR-independent STAT3 phosphorylation we used the Janus-activated kinase (JAK) inhibitor AG490 (Fig. 3*B*). AG490 had marginal effects on STAT3 phosphorylation in 2 of the 3 cell lines exhibiting EGFR-independent STAT3-Y705 phosphorylation, although it inhibited interleukin 6-induced STAT3 phosphorylation in K562 erythroleukemia cells (Fig. 3*C*). A combination of AG1478 and AG490 did not reveal additive effects on EGFR-independent STAT3-Y705 phosphorylation. Furthermore, a triple combination of AG1478/AG490 and SRC kinase inhibitors (PP1 or PP2) did not significantly affect EGFR-independent steady-state STAT3 phosphorylation (data not shown). Taken together, these results point to the existence of EGFR-dependent and EGFR/JAK-independent pathways that cooperatively induce STAT3 tyrosine phosphorylation in SCCs.

DNA Binding of STAT3 in Normal and Malignant Keratinocytes. To additionally define the effect of EGFR activation on STAT3 activity in SCCs we performed electrophoretic mobility shift assays using oligonucleotides containing a STAT3 DNA binding motif (Fig. 4). These experiments were done using cell extracts prepared in the presence and absence of exogenous EGF and the EGFR inhibitor AG1478. Only malignant tumor cell extracts produced STAT3:DNA complexes, and this phenomenon was restricted to extracts from EGF-treated cultures. SCC12 cells were a notable exception, because no DNA binding activity was observed either in the presence or absence of exogenous EGF, although these cells showed STAT3-Y705 phosphorylation under these conditions (Fig. 2). As expected, STAT3:DNA complexes were absent in both HaCaT cells and normal keratinocytes regardless of treatment with EGF. Interestingly, tumor

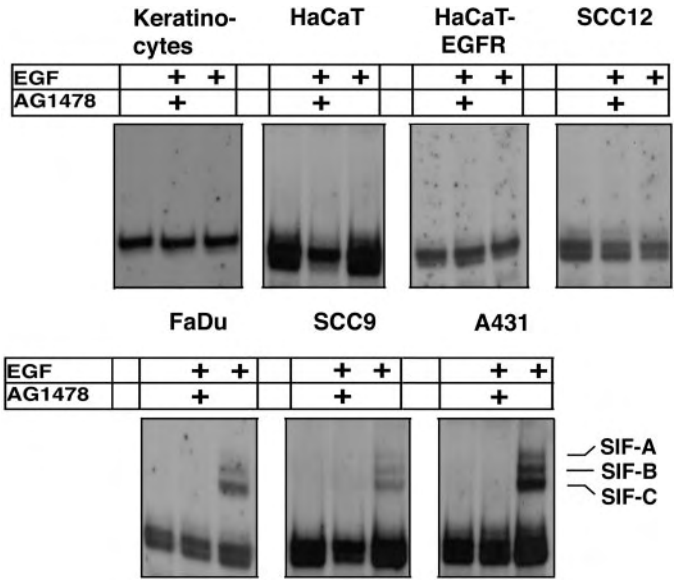

Fig. 4. DNA binding of signal transducer and activator of transcription (STAT)3 in SCC lines. Cell lines were treated with epidermal growth factor (*EGF*; 10 ng/ml) in the presence and absence of AG1478 (10 μM) as indicated. Cell extracts were prepared and subjected to electrophoretic mobility shift assays with ^{32}P-labeled STAT3-specific probes. The position of protein/DNA complexes is indicated. The nomenclature of the complexes (*SIF-A, -B,* and *-C*) refers to the designations given previously (26). DNA binding activity of STAT3 was restricted to EGF-treated FaDu, SCC9, and A431 cultures.

cells exhibiting EGFR-independent STAT3-Y705 phosphorylation did not exhibit DNA binding in the absence of exogenous EGF. This result is consistent with the notion that, in malignant keratinocytes, STAT3 phosphorylation on Y705 is not sufficient for transactivation potential.

Absence of STAT3 Phosphorylation in HaCaT Cells Overexpressing the EGFR. Next, we asked the question of whether overexpressing the EGFR in HaCaT cells affects STAT3 tyrosine phosphorylation. To this end, we generated HaCaT cells with levels of EGFR expression similar to those observed in SCCs (HaCaT-EGFR; Fig. 5). Although EGFR phosphorylation in these cells was markedly higher than in parental HaCaT cells and comparable with those observed in SCC9 or SCC12 cells (Fig. 1), neither STAT3-Y705 phosphorylation nor DNA binding activity (Fig. 4) was observed in these cells either in the presence or absence of exogenous EGF.

Inhibition of STAT3 Phosphorylation in Normal and Malignant Keratinocytes by EGFR-Dependent MEK Activity. This result raised the issue of whether, in HaCaT keratinocytes, STAT3 tyrosine phosphorylation can be induced by any means. To this end, we first used HaCaT cells conditionally overexpressing a dominant-negative but phosphorylatable STAT3 construct (STAT3D) as described earlier (15, 27). As shown in Fig. 6*A*, upon overexpression of STAT3D in HaCaT cells, robust STAT3-Y705 phosphorylation was observed. By contrast and as expected, conditional overexpression of a dominant-negative construct in which Y705 had been replaced by phenylalanine (STAT3F; Ref. 27) did not result in STAT3-Y705 phosphorylation under these conditions. Next, we assessed whether STAT3 expressed at physiological levels could be phosphorylated on Y705 by using the MEK inhibitor U0126 (Fig. 6*B*). This was done based on previous reports, which demonstrated that blocking MEK enhances interleukin 6-dependent STAT3 tyrosine phosphorylation in HepG2 hepatoma and MM6 myeloma cells (28, 29). Consistent with these earlier reports pharmacological inhibition of MEK activity with the U0126 compound was accompanied not only by reduced phosphorylation of MAPK but also by increased STAT3 Y705 phosphorylation in HaCaT cells. Interestingly, this effect was obvious only in the presence of exogenous EGF. Similarly, blocking MEK activity in SCC9, FaDu, SCC12, and A431 cells with U0126 was associated with enhanced levels of STAT3 Y705 phosphorylation in response to exogenous EGF. To ascertain that this effect was due to inhibition of MEK rather than nonspecific effects of U0126, we used HaCaT keratinocytes engineered to express dominant-negative MEK in an inducible fash-

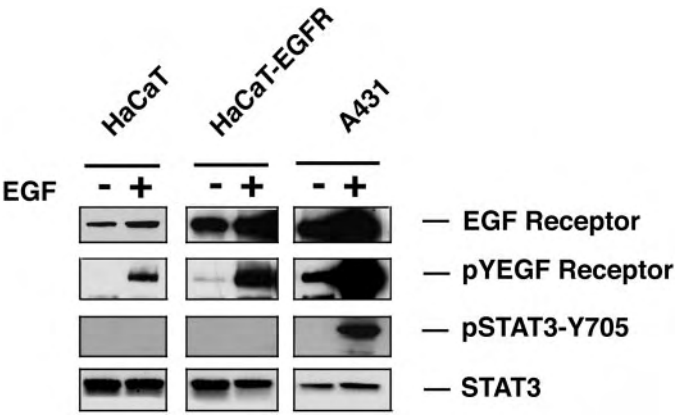

Fig. 5. Effect of forced expression of the epidermal growth factor (*EGF*) receptor on signal transducer and activator of transcription (*STAT*)3 phosphorylation in HaCaT cells. Expression and autophosphorylation states of the EGF receptor were assessed in HaCaT cells, HaCaT cells engineered to express high levels of the EGF receptor, and A431 cells as controls. In all cases, cells were treated with EGF for 15 min before preparing cell extracts and Western blot analyses with antibodies as indicated.

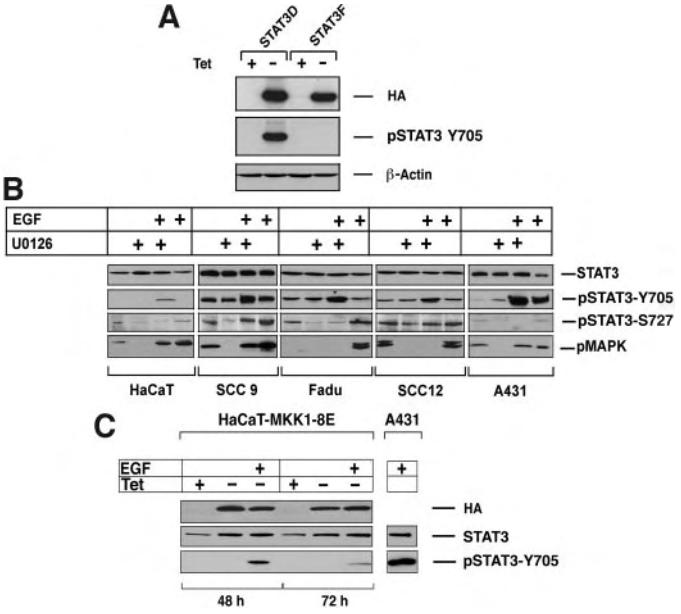

Fig. 6. Modifiers of signal transducer and activator of transcription (*STAT*)3 tyrosine phosphorylation in immortalized and malignant epithelial cells. *A* demonstrates that induced expression of a phosphorylatable STAT3 construct (*STAT3D*) in HaCaT keratinocytes is associated with constitutive tyrosine phosphorylation of this construct. Induction of the transgene by removal of tetracycline (*Tet*) from the culture medium was detected by Western blot analysis using an antibody against the hemagglutinin tag. Phosphorylation of STAT3 was probed using an antibody that specifically recognizes STAT3-Y705. As a loading control the expression of β-actin was determined in the same samples. A nonphosphorylatable STAT3 construct included as a negative control (*STAT3F*) was not phosphorylated. *B* shows induction of epidermal growth factor (*EGF*) receptor-dependent STAT3-Y705 phosphorylation in immortalized HaCaT cells and in squamous cell carcinoma (*SCC*) cells by treatment of cells with the mitogen-activated protein kinase kinase inhibitor U0126. *C* demonstrates induction of EGF receptor-dependent STAT3-Y705 phosphorylation in HaCaT cells expressing a dominant-negative MEK1 construct (MKK1–8E). STAT3 phosphorylation was detected upon induction of the hemagglutinin (*HA*)-tagged transgene by removal of tetracycline from the culture medium and was strictly dependent upon the presence of exogenous EGF. Expression and tyrosine phosphorylation of STAT3 in control A431 cells are also shown.

ion as described previously (15). Induction of the transgene in these cells also led to STAT3-Y705 phosphorylation in a strictly EGFR-dependent manner (Fig. 6*C*). These results demonstrate that EGFR activation can elicit STAT3 phosphorylation on Y705 in HaCaT cells. However, for this effect to take place concomitant MEK activation needs to be inhibited.

EGFR-Dependent STAT3 Serine Phosphorylation. It has been described previously that EGFR activation leads to phosphorylation of STAT3 on S727 through a MAPK-dependent pathway (28). Therefore, we assessed patterns of dual phosphorylation of STAT3 on Y705 and S727 in the presence of EGF in SCCs (Fig. 6*B*). Because S727 phosphorylation appears to be induced by MAPK in other cell systems we also tested the effect of the MEK inhibitor U0126 on S727 STAT3 phosphorylation. The results of this analysis showed S727 phosphorylation in all of the cell lines in the absence of exogenous EGF. Addition of EGF led to increased S727 phosphorylation in SCC9 and FaDu cells but not in HaCaT, SCC12, or A431 cells. Treatment with U0126 moderately reduced but did not abrogate S727 phosphorylation in HaCaT, SCC9, FaDu, and A431 cells in the presence and absence of exogenous EGF. By contrast, only marginal effects of U0126 were observed in SCC12 cells. These results suggest that STAT3-S727 phosphorylation in SCC lines is, in part, dependent on MEK activity. Yet, no obvious relationship between EGF-dependent STAT3-S727 phosphorylation and DNA binding of STAT3 emerged in the panel of cell lines investigated here. Specifically, in A431 cells, EGF induced robust DNA binding, yet STAT3-S727 phosphorylation was not affected. In addition, comparatively high levels of STAT3-S727 phosphorylation were observed in SCC12 cells, which revealed no STAT3/DNA binding activity either in the absence or presence of exogenous EGF. We conclude that EGFR phosphorylation is likely to contribute a signal distinct from Y705 and S727 phosphorylation that is necessary to acquire STAT3 DNA binding competence.

DISCUSSION

The present study was designed to test the hypothesis that STAT3 activation is a tumor-associated phenomenon associated with deregulated EGFR signaling in SCCs. Whereas the results obtained strongly support this contention they also raise several unexpected issues about STAT3 activation in normal and malignant keratinocytes. First and foremost we observed that dual STAT3 phosphorylation on Y705 and S727 was not sufficient to induce STAT3 DNA binding in this cell type. These results are reminiscent of a recent study by Bild *et al.* (30) who demonstrated that, in addition to phosphorylation, endocytotic transport of STAT3, presumably complexed with the activated EGFR, is a necessary prerequisite for nuclear import, DNA binding, and transcriptional activity in NIH3T3 cells engineered to overexpress the EGFR.

Another rather unexpected result of this study related to the presence of pSTAT3-Y705 in growth factor-starved malignant epithelial cells. Furthermore, STAT3-Y705 phosphorylation persisted in these cells in the presence of the EGFR inhibitor AG1478. During preparation of this article a similar observation was reported using a different set of head and neck SCCs (31). In that report EGFR-independent STAT3-Y705 phosphorylation was ascribed to autocrine interleukin 6/JAK-dependent signaling. Our results provide only limited support for this conclusion, because treatment with the JAK inhibitor AG490 reduced pSTAT3-Y705 content in only one (SCC12) of the three cell lines tested. By contrast, AG490 had no detectable effect on pSTAT3-Y705 content in SCC9 and FaDu cells. Similarly, the kinase responsible for STAT3-Y705 phosphorylation in these SCC cells is not a SRC-like kinase, because SRC inhibitors (PP1/PP2) had only negligible effects on EGFR-independent STAT3Y705 phosphorylation.[3] Thus, we have excluded the three major pathways known to contribute to STAT3 tyrosine phosphorylation, *i.e.,* the EGFR, a SRC-like kinase, and gp130/JAK in the maintenance of STAT3 phosphorylation in the growth factor-starved state of SCC in cell culture. Identification of the kinase responsible for this constitutive STAT3-Y705 phosphorylation awaits future studies.

A third observation of interest relates to the finding that EGFR-dependent activation of MEK/MAPK signaling negatively regulates STAT3-Y705 phosphorylation in HaCaT cells and in SCCs. Although this phenomenon has been described before in another cell type (28), the involvement of MEK in those systems was implied only by using pharmacological MEK inhibitors, which are known to also inhibit other members of the MEK family, for example, MEK5 (32). By using HaCaT cells conditionally overexpressing dominant-negative MEK we were able to provide independent evidence confirming that EGFR-dependent MEK activation serves to suppress EGFR-dependent STAT3-Y705 phosphorylation in HaCaT cells. Moreover, we used cells expressing physiological levels of STAT3 to make this observation. This experimental detail is of considerable importance, because we observed that forced overexpression of STAT3 led to robust Y705 phosphorylation irrespective of regulatory influences (see Fig. 6*A*).

In conclusion, the results of the present study highlight complex regulation of STAT3 activation in SCC lines. Of largest practical import is our finding that STAT3 activation is a tumor-associated phenomenon in this cell system and strictly dependent on EGFR activation. Furthermore, our results illuminate the importance of distinguishing STAT3 phosphorylation events from DNA binding activities. Although STAT3 tyrosine phosphorylation appears to be necessary for biological activity, only DNA binding and subsequent transcriptional effects are likely to contribute to STAT3 function relevant to the malignant phenotype.

ACKNOWLEDGMENTS

We thank Dr. James G. Rheinwald for providing SCC cell lines, Dr. Norbert Fusenig for providing HaCaT cells, and Sharon Connelly for technical assistance.

REFERENCES

1. Rodeck U, Jost M, Kari C, et al. EGF-R dependent regulation of keratinocyte survival. J Cell Sci 1997;110:113–21.
2. Stoll SW, Benedict M, Mitra R, Hiniker A, Elder JT, Nunez G. EGF receptor signaling inhibits keratinocyte apoptosis: evidence for mediation by Bcl-XL. Oncogene 1998;16:1493–9.
3. Kijima T, Niwa H, Steinman RA, et al. STAT3 activation abrogates growth factor dependence and contributes to head and neck squamous cell carcinoma tumor growth in vivo. Cell Growth Diff 2002;13:355–62.
4. Jost M, Gasparro FP, Jensen PJ, Rodeck U. Keratinocyte apoptosis induced by UVB radiation and CD95 ligation - differential protection through EGFR activation and Bcl-xL expression. J Investig Dermatol 2001;116:860–6.
5. Jost M, Huggett TM, Kari C, Rodeck U. Matrix-independent survival of human keratinocytes through an EGF receptor/MAPK-kinase-dependent pathway. Mol Biol Cell 2001;12:1519–27.
6. Daly JM, Jannot CB, Beerli RR, Graus-Porta D, Maurer FG, Hynes NE. Neu differentiation factor induces ErbB2 down-regulation and apoptosis of ErbB2-overexpressing breast tumor cells. Cancer Res 1997;57:3804–11.
7. Yu D, Jing T, Liu B, et al. Overexpression of ErbB2 blocks Taxol-induced apoptosis by upregulation of p21Cip1, which inhibits p34Cdc2 kinase. Mol Cell 1998;2:581–91.
8. Roh H, Pippin J, Drebin JA. Down-regulation of HER2/neu expression induces apoptosis in human cancer cells that overexpress HER2/neu. Cancer Res 2000;60:560–5.
9. Lazar H, Baltzer A, Gimmi C, Marti A, Jaggi R. Over-expression of erbB-2/neu is paralleled by inhibition of mouse-mammary-epithelial-cell differentiation and developmental apoptosis. Int J Cancer 2000;85:578–83.
10. Zhou BP, Hu MC, Miller SA, et al. HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NF-kappaB pathway. J Biol Chem 2000;275:8027–31.

[3] M. R. D. Quadros, unpublished observations.

11. Rodeck U, Jost M, DuHadaway J, et al. Regulation of Bcl-xL expression in human keratinocytes by cell-substratum adhesion and the epidermal growth factor receptor. Proc Natl Acad Sci USA 1997;94:5067–72.
12. Jost M, Class R, Kari C, Jensen PJ, Rodeck U. A central role of Bcl-X(L) in the regulation of keratinocyte survival by autocrine EGFR ligands. J Investig Dermatol 1999;112:443–9.
13. Dlugosz AA, Hansen L, Cheng C, et al. Targeted disruption of the epidermal growth factor receptor impairs growth of squamous papillomas expressing the v-ras(Ha) oncogene but does not block in vitro keratinocyte responses to oncogenic ras. Cancer Res 1997;57:3180–8.
14. Huang SM, Bock JM, Harari PM. Epidermal growth factor receptor blockade with C225 modulates proliferation, apoptosis, and radiosensitivity in squamous cell carcinomas of the head and neck. Cancer Res 1999;59:1935–40.
15. Jost M, Huggett TM, Kari C, Boise LH, Rodeck U. Epidermal growth factor receptor-dependent control of keratinocyte survival and Bcl-xL expression through a MEK-dependent pathway. J Biol Chem 2001;276:6320–6.
16. Gilmore AP, Valentijn AJ, Wang P, et al. Activation of BAD by therapeutic inhibition of epidermal growth factor receptor and transactivation by insulin-like growth factor receptor. J Biol Chem 2002;277:27643–50.
17. Bromberg JF, Horvath CM, Besser D, Lathem WW, Darnell JE Jr. Stat3 activation is required for cellular transformation by v-src. Mol Cell Biol 1998;18:2553–8.
18. Bromberg JF, Wrzeszczynska MH, Devgan G, et al. Stat3 as an oncogene. Cell 1999;98:295–303.
19. Grandis JR, Drenning SD, Zeng Q, et al. Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis in vivo. Proc Natl Acad Sci USA 2000;97:4227–32.
20. Rodeck U, Herlyn M, Herlyn D, et al. Tumor growth modulation by a monoclonal antibody to the epidermal growth factor receptor: immunologically mediated and effector cell-independent effects. Cancer Res 1987;47:3692–6.
21. Murthy U, Basu A, Rodeck U, Herlyn M, Ross AH, Das M. Binding of an antagonistic monoclonal antibody to an intact and fragmented EGF-receptor polypeptide. Arch Biochem Biophys 1987;252:549–60.
22. McNeill H, Jensen PJ. A high-affinity receptor for urokinase plasminogen activator on human keratinocytes: characterization and potential modulation during migration. Cell Regul 1990;1:843–52.
23. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 1988;106:761–71.
24. Rodeck U, Herlyn M, Menssen HD, Furlanetto RW, Koprowsk H. Metastatic but not primary melanoma cell lines grow in vitro independently of exogenous growth factors. Int J Cancer 1987;40:687–90.
25. Jost M, Kari C, Rodeck U. An episomal vector for stable tetracycline-regulated gene expression. Nucleic Acids Res 1997;25:3131–4.
26. Zhong Z, Wen Z, Darnell JE Jr. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. Science 1994;264:95–8.
27. Nakajima K, Yamanaka Y, Nakae K, et al. A central role for Stat3 in IL-6-induced regulation of growth and differentiation in M1 leukemia cells. EMBO J 1996;15:3651–8.
28. Chung J, Uchida E, Grammer TC, Blenis J. STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. Mol Cell Biol 1997;17:6508–16.
29. Sengupta TK, Talbot ES, Scherle PA, Ivashkiv LB. Rapid inhibition of interleukin-6 signaling and Stat3 activation mediated by mitogen-activated protein kinases. Proc Natl Acad Sci USA 1998;95:11107–12.
30. Bild AH, Turkson J, Jove R. Cytoplasmic transport of Stat3 by receptor-mediated endocytosis. EMBO J 2002;21:3255–63.
31. Sriuranpong V, Park JI, Amornphimoltham P, Patel V, Nelkin BD, Gutkind JS. Epidermal growth factor receptor-independent constitutive activation of STAT3 in head and neck squamous cell carcinoma is mediated by the autocrine/paracrine stimulation of the interleukin 6/gp130 cytokine system. Cancer Res 2003;63:2948–56.
32. Kamakura S, Moriguchi T, Nishida E. Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of a signaling pathway to the nucleus. J Biol Chem 1999;274:26563–71.

[CANCER RESEARCH 63, 1–5, January 1, 2003]

Perspectives in Cancer Research

Targeting the Epidermal Growth Factor Receptor in Cancer: Apoptosis Takes Center Stage[1]

Csaba Kari, Tung O. Chan, Marlene Rocha de Quadros, and Ulrich Rodeck[2]

Department of Dermatology and Cutaneous Biology [C. K., M. R. d. Q., U. R.], and the Kimmel Cancer Center [T. O. C., U. R.], Thomas Jefferson University, Philadelphia, Pennsylvania 19130

Abstract

Aberrant activation of the epidermal growth factor receptor (EGFR) is frequently observed in neoplasia, notably in tumors of epithelial origin. Attempts to treat such tumors with EGFR antagonists have met with remarkable initial successes, particularly when EGFR antagonists were used in combination with chemotherapy or ionizing radiation. Considering the almost ubiquitous expression of the EGFR in normal epithelial tissues, these clinical trials also revealed a surprisingly low rate of adverse side effects associated with EGFR blockade. This review highlights anti-apoptotic effects of EGFR activation as they relate to therapeutic efficacy of EGFR blockade. We introduce the concept that control of cell survival through EGFR activation is conditional in the sense that it is rate limiting to tumor cell survival but not to survival of normal epithelial cells. Specifically, normal epithelial cells are provided with a full complement of physiological cell-cell contacts and cell-matrix interactions that lessen their dependence on survival signals provided by the EGFR. By contrast, malignant tumor cells faced with inadequate cell-matrix contacts critically depend on EGFR activation for survival, rendering them more susceptible to apoptosis induction by EGFR blockade. Redundant control of cell survival by the EGFR and extracellular matrix/cell adhesion receptors is enabled, in part, by shared signal transduction pathways that control expression and activation states of members of the Bcl-2 family of apoptosis regulators.

Introduction

Multicellular organisms critically depend on efficient intercellular communication. This information exchange instructs cell fate during development, maintains homeostasis in the mature organism, and coordinates appropriate responses to challenges of the homeostatic state. Information flow is enabled by (1) extracellular matrix components binding to adhesion receptors (2), cell-cell contact between neighboring cells (3), and soluble mediators (growth factors, cytokines, hormones) that engage specific cell surface receptors. Signals emanating from matrix and cell contact receptors provide positional information as they are spatially confined. By contrast, soluble mediators can act at a distance whether by distribution through the blood stream or by diffusion. Thus, soluble factors provide a rapid and versatile response to disturbances of the homeostatic state. This is perhaps best illustrated by the wound healing process because it is characterized by successive waves of soluble mediators with complex effects on multiple cell types in the wound bed. The burst of biological activities during wound healing is limited in time and space eventually leading to the status quo ante.

By contrast, malignant tumors frequently show constitutive deregulation of growth factor receptor signaling. In fact, among the first viral oncogenes to be described were platelet-derived growth factor B (1) and a constitutively active form of the EGFR[3] (2, 3), the subject of this review. Deregulation of growth factor receptor signaling is not limited to virally induced tumors but occurs across a wide spectrum of spontaneous tumors of diverse tissue origin.

The EGFR Family in Epithelial Neoplasia. In epithelial malignancies, the ErbB family of receptors and their ligands are prominent targets of genetic or epigenetic alterations, which frequently lead to their inappropriate activation. The EGFR (EGFR/c-ErbB1/Her-1) is one of four members of the ErbB family of type-1-tyrosine kinases, which also includes ErbB2/Her2, Her3, and Her4. Six ligands of the EGFR are known to activate the tyrosine kinase moiety of the receptor, and the signal strength of EGFR activation may be amplified by heterodimerization with other members of the ErbB family, notably ErbB2. Epidemiological evidence accrued over the last 20 years in human tumors buttresses the notion that aberrant EGFR expression and signaling contribute to the development of multiple epithelial malignancies in humans. These include squamous carcinomas of the skin and breast cancer among others (4–9). In several epithelial tumor systems, EGFR alterations occur at advanced stages of malignancy characterized by metastatic competence (7, 10). In certain tumors, including some glioblastomas and, as shown recently, breast cancers, a truncated EGFR lacking a portion of the extracellular domain is expressed (EGFRvIII). This altered version of the EGFR appears to be constitutively active and is oncogenic in 3T3 transformation assays (11–13). In addition to the EGFR, ErbB2 is well recognized as a proto-oncogene consistent with a broad role of ErbB receptors in malignant transformation. Because of space considerations, we focus the following discussion to the EGFR/ErbB1; for in-depth reviews of the interdependent roles of ErbB1 and ErbB2 in oncogenesis, please refer to Refs. 14, 15.

EGFR Signaling as It Relates to the Malignant Phenotype. In normal or malignant skin epithelial cells, EGFR activation drives cell cycle progression (16, 17), supports migration and invasion (2, 18, 19), and affects differentiation (20, 21). Similarly, EGFR activation supports scattering and invasion of breast epithelial cells in three-dimensional culture associated with loss of cell polarization and other features of epithelial differentiation (22). Any of these effects alone or in combination may contribute to the malignant phenotype. However, in this review we will focus on EGFR-dependent cell survival as we consider this phenomenon to be critical to cancer biology and highly relevant to recent attempts to treat epithelial cancer by blocking the EGFR (for reviews of recent clinical trials see Refs. 23, 24). We will describe evidence that EGFR blockade enhances apoptosis susceptibility in conditions of cellular stress, outline molecular mechanisms

Received 7/31/02; accepted 10/31/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported, in part, by a grant from the W. W. Smith Foundation, NIH Grant CA81008, and United States Department of the Army Grant DAMD17-02-1-0216 (to U. R.).

[2] To whom requests for reprints should be addressed, at Thomas Jefferson University, 233 South 10th Street, BLSB 319, Philadelphia, PA 19107. Phone/Fax: (215) 503-5622; E-mail: Ulrich.Rodeck@mail.tju.edu.

[3] The abbreviations used are: EGFR, epidermal growth factor receptor; MAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MEK, MAP kinase kinase, PI3k, phosphatidylinositol 3′-kinase, PKB, protein kinase B (synonymous with AKT); STAT, signal transducer and activator of transcription.

involved in this phenomenon, and discuss the results of recent preclinical and clinical studies of EGFR blockade in this context.

Cell Death by EGFR Blockade. The development of EGFR specific inhibitors enabled the rigorous study of the relative contribution of EGFR signaling to cellular phenotypes. Two types of inhibitors have been produced distinguished by different modes of action. MAbs to the external domain of the EGFR have been developed that disrupt ligand binding to the receptor and subsequent signal transduction. Three EGFR-specific blocking antibodies have been characterized in greater detail *in vitro* and are presently used in clinical studies; these are mAbC225 (ERBITUX/cetuximab), mAb425 (EMD72000) and the human mAb ABX-EGF. In contrast to mAbs, small molecular weight inhibitors of the EGFR block the ATP acceptor site (lysine 721) located within the intracellular kinase domain. Blockade of ATP loading prevents phosphate transfer to tyrosine residues and, thus, autophosphorylation of the EGFR and subsequent signal transduction. Examples for this type of inhibitor are EGFR selective tyrphostins (25), including the ZD1839 compound (Iressa; Ref. 24).

A role of EGFR activation in support of epithelial cell survival was first suggested by the observation that treatment with mAbC225 induces spontaneous apoptosis of the colorectal carcinoma cell line DiFi (26); the term spontaneous apoptosis is used here to describe apoptosis in the absence of obvious cellular stress. However, mAbC225 treatment rarely induces spontaneous apoptosis in malignant epithelial cells other than the DiFi cell line (23). Similarly, normal keratinocytes and squamous carcinoma cells tolerate EFGR blockade by treatment with mAb425 as long they are maintained under homeostatic culture conditions *in vitro* (27).

Dramatically different results were obtained when cells were subjected to cellular stress *in vitro*. For example, in normal keratinocytes, EGFR blockade induces large-scale apoptosis when cells are passaged (27). In addition, EGFR blockade sensitizes human keratinocytes to apoptosis induction by UV radiation (28). In the preclinical setting, mAbC225 has been shown to enhance apoptosis of malignant epithelial cells induced by many cellular stressors, including ionizing radiation and various chemotherapeutic drugs (for review see Ref. 23). Collectively, these results indicate an important survival function of the EGFR in conditions of cellular stress for both normal (*e.g.,* primary keratinocytes) and transformed epithelial cells. Yet, they do not explain why systemic EGFR blockade is well tolerated in experimental animals or patients. Recent experiments focusing on anchorage-independent cell survival may help to resolve this issue.

Matrix-independent Cell Survival Enabled by EGFR Activation. Frisch and Francis (29) were among the first to realize that loss of matrix attachment leads not only to growth arrest but also to death of normal epithelial cells (immortalized keratinocytes, MDCK cells) by apoptosis and have termed this phenomenon anoikis. Other cell types, including mammary epithelial cells (30), endothelial cells (31), and some fibroblast cell lines (32), are similarly prone to anoikis when adhesion receptors are disengaged for prolonged periods of time. The dependence on matrix interaction for cell survival is believed to provide an important safeguard against inappropriate expansion and metastatic spread of normal epithelial cells. Consistent with this notion, anoikis-resistant tumor cells generally metastasize at high rates in animal models (33, 34). This leaves the question how tumor cells achieve anchorage-independent survival.

Recent work has demonstrated that activation of the EGFR provides a measure of protection against anoikis in the suspended state even to normal cells such as keratinocytes (35) or mammary epithelial cells (36). Importantly, EGFR blockade sensitizes normal epithelial cells to apoptosis induction in the suspended but to a much lesser extent in the attached state (35, 37). This observation extends to malignant epithelial cells as 6 of 7 tested squamous carcinoma cell lines die at faster rates upon EGFR blockade in the suspended but not the attached state.[4] Taken together, these results suggest that adhesion receptors and growth factor receptors such as the EGFR provide complementary and functionally redundant survival signals to epithelial cells. Because of this redundancy, normal cells that receive the full complement of physiological matrix-derived survival signals are relatively resistant to apoptosis induced by EGFR blockade (Fig. 1*A*). By contrast, malignant tumor cells in transit or at sites with inadequate matrix composition have little tolerance for EGFR blockade because adhesion receptor signaling is reduced or absent (Fig. 1*B*). These cells are also much less likely to resist additional stress caused by either chemotherapeutic drugs or radiation therapy as compared with their normal counterparts.

It should be noted that the EGFR is but one of several receptor tyrosine kinases that are known to alleviate anoikis, including the insulin-like growth factor-1-receptor (38) and Met (39). Similarly, up-regulation of integrin-dependent signal transducers, including focal adhesion kinase (38, 40) and integrin-linked kinase (41–44) alleviates anoikis in certain tumor cells. Thus, a diverse array of kinases modulates anoikis sensitivity. Yet, among these kinases, the EGFR and its homologue, ErbB2 are most frequently deregulated in epithelial malignancies.

A series of recent studies using organotypic cultures representing breast acini further highlights the complex regulation of cell migration and cell survival by cell matrix adhesion and EGFR/ErbB2 signaling. Specifically, engagement of the β4 integrin subunit confers apoptosis resistance to immortalized breast epithelial cells in three-dimensional culture on reconstituted basement membranes (45). Conversely, disruption of integrin-dependent cell polarization renders these cells more susceptible to apoptosis induction. Forced expression of either Bcl-2 or Bcl-x_L (46) or activation of c-ErbB2 (47) attenuates apoptosis of breast epithelial cells that have lost contact to basement membranes under these culture conditions. These studies are consistent with the notion that ErbB family members serve a dual role during cell invasion. Not only do they induce migration and invasion of breast epithelial cells in organotypic cultures (22), but they also sustain survival of cells that have lost positional survival signals. In this context, it remains to be determined whether the reduction in the diversity of antiapoptotic signals during migration and invasion is a consequence of epithelial-mesenchymal transitions that accompany EGFR activation in select tumor cells of epithelial origin (48). Specifically, loss of cell/cell and cell/matrix adhesion during epithelial-mesenchymal transitions may be induced by EGFR activation and necessitate increased reliance on EGFR activation for cell survival.

Modulating Apoptosis Susceptibility by EGFR Activation: Molecular Mechanisms and Targets. Several signal transduction pathways have been implicated in EGFR-dependent cell survival as it relates to the anchorage-independent state. Primarily, these include the RAS/RAF/MEK/MAPK cascade and STAT3-dependent signaling events.

EGFR-dependent MEK/MAPK signaling is essential to survival of normal or immortalized keratinocytes in the absence of matrix-derived signals (49). This conclusion is supported by the findings that (*a*) MAPK phosphorylation is markedly reduced in suspension culture, (*b*) EGF treatment leads to robust and sustained MAPK phosphorylation in suspension culture, and (*c*) inhibiting MEK by either PD98059 or a dominant negative expression construct induces apoptosis/anoikis in keratinocytes. Similarly, EGFR-dependent survival of fibroblasts (32) and mammary epithelial cells (36) appears to be mediated, in part, by MEK/MAPK-dependent signals. At least two

[4] U. Rodeck, unpublished observation.

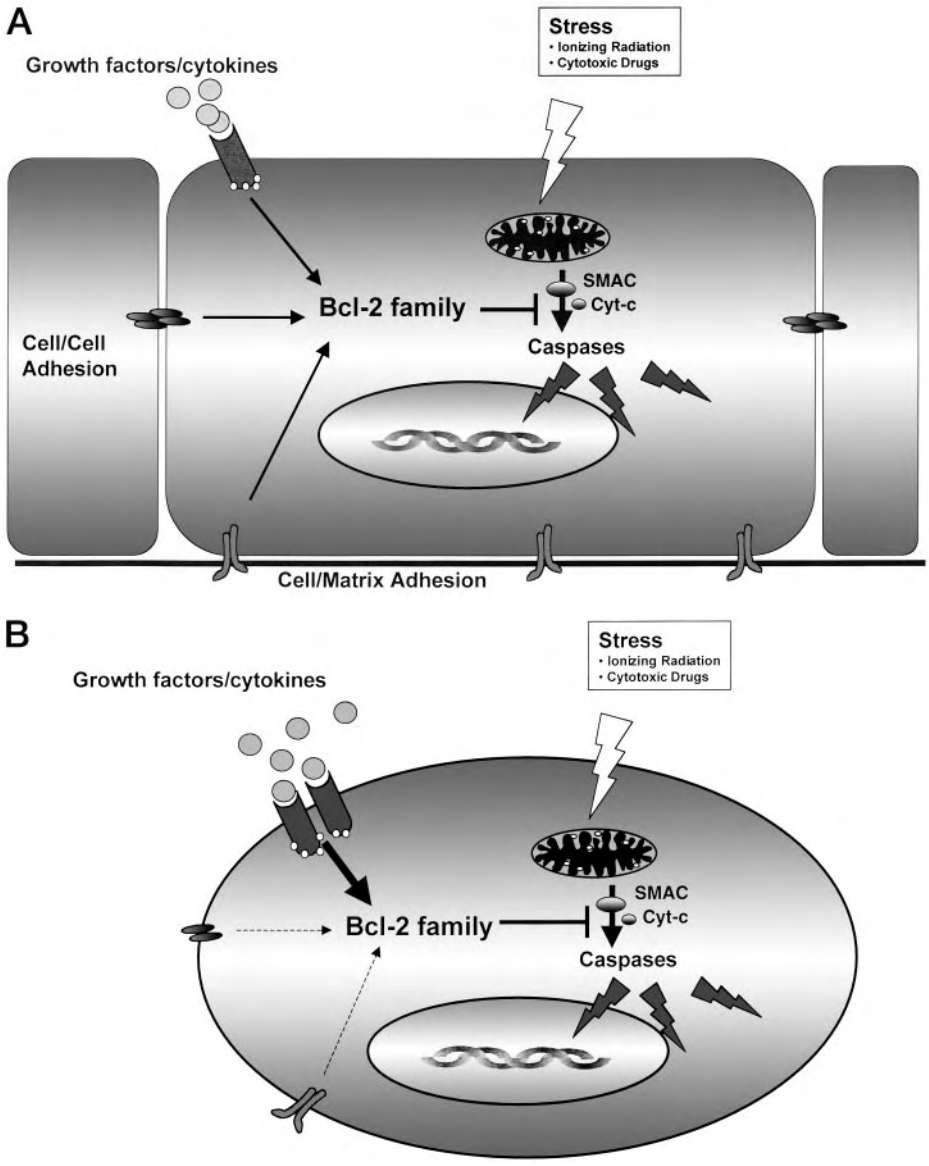

Fig. 1. Differential apoptosis susceptibility of normal epithelial tissues (*A*) and tumor cells during invasion and metastasis (*B*). In normal tissues, multiple signaling pathways converge on Bcl-2 family members to enhance protection against apoptosis induced by cellular stress. Cooperative signaling through direct cell-cell contact, interaction with extracellular matrix components, and soluble mediators provides complementary and redundant antiapoptotic signals. By contrast, cancer cells in transit or at sites with inappropriate matrix composition rely heavily on growth factors such as EGFR ligands for survival. EGFR blockade will critically weaken tumor cell resistance to apoptosis induction in inhospitable microenvironments whereas normal cells maintain a measure of apoptosis resistance through positional signals.

MAPK targets relevant for cell survival in the anchorage-independent state have been identified. We and others observed that, in keratinocytes, EGFR-dependent MAPK activation affects the balance of members of the Bcl-2 family of proteins that control apoptosis susceptibility. Specifically, EGFR-mediated MAPK activation is required for high level expression of Bcl-x_L, an antiapoptotic member of the Bcl-2 family of proteins (35, 49, 50). By contrast, expression of the proapoptotic Bcl-family members Bad, Bak, and Bax is not affected by EGFR activation in this cell system. Similarly, Bcl-x_L expression and survival of MDCK cells in the anchorage-independent state depend on EGFR signaling (51). Finally, EGFR-dependent Bcl-x_L expression has been implicated in protection of glioblastoma cells expressing EGFRvIII against cisplatin-induced apoptosis (52, 53), although the role of MEK/MAPK signaling in this phenomenon remains to be determined. A second EGFR/MAPK-dependent survival mechanism targets the proapoptotic Bcl-2 family member BAD in mammary epithelial cells. Specifically, EGFR blockade prevents MAPK-dependent BAD phosphorylation on serines 112 and 155 (36). Phosphorylation is a prerequisite for sequestration and functional inactivation of BAD (54–56). Taken together, these results indicate that EGFR-dependent MAPK signaling targets apoptosis regulators of the Bcl-2 family not only by affecting their expression levels but also by posttranslational modification. The importance of MEK/MAPK signals in anchorage-independent tumor cell survival is underscored by the observation that pharmacological inhibitors of MEK restore anoikis sensitivity to human breast cancer cells (57).

Activation of the STAT3 provides a second well-documented EGFR-dependent survival mechanism (for review see Ref. 58). STAT3 activation contributes to expression of Bcl-x_L in myeloma cells (59) and to survival and Bcl-x_L expression of certain squamous cell carcinomas (60, 61). By contrast, in normal or immortalized keratinocytes, EGFR activation does not significantly contribute to STAT3 phosphorylation, and suppression of STAT3 activity by dominant negative constructs affects neither survival of nor Bcl-x_L ex-

pression by these cells (49). These results raise the intriguing question whether EGFR-dependent STAT3 activation is a tumor-associated event suitable to therapeutic targeting.

In certain experimental settings, EGFR activation is associated with activation of the PI3k/AKT survival pathway (62–64) or nuclear factor κB (65, 66). As discussed for STAT3, AKT/PKB phosphorylation through EGFR engagement may be restricted to certain tumor cells or experimental conditions as it is not observed in normal or immortalized keratinocytes (49). However, forced expression of oncogenic RAS (Ha-RasV12) enables EGFR-dependent AKT phosphorylation in keratinocytes in forced suspension culture (67). Similarly, forced overexpression of the tumor-associated EGFRvIII variant in fibroblasts is associated with strong activation of PI3k (13). Clearly, additional studies are necessary to distinguish physiological and tumor-associated effects of EGFR activation on signaling pathways.

Of note, EGFR-mediated and extracellular matrix-induced signaling events converge on regulating expression levels and functional states of Bcl-2 family members. For example, in CHO cells, $\alpha 5/\beta 1$ integrin engagement up-regulates Bcl-2 expression in a Ras/PI3k-dependent manner (68). Furthermore, matrix adhesion prevents functional activation of the proapoptotic Bcl-2 family member Bax in mammary epithelial cells in a focal adhesion kinase and PI3k-dependent manner (69). Finally, activation of E-cadherin reportedly enhances survival of squamous carcinoma cells in a three-dimensional culture model, and this effect is paralleled the up-regulation of Bcl-2 (70). Collectively, these results reinforce the notion of redundancy in survival signals emanating from cell-cell, cell-matrix, and growth factor receptors.

Conclusions

Recent work has firmly established that EGFR activation supports survival of nontransformed and transformed epithelial cells. However, the survival function of the EGFR appears to be conditional in the sense that, particularly, tumor cells faced with inappropriate or inadequate cell-matrix contacts critically depend on EGFR activation for survival. This circumstance may explain preferential killing of tumor cells by use of EGFR blocking agents in the adjuvant setting in tumor patients. Additional work is needed to understand the coordinate and potentially redundant regulation of cell survival by activation of the EGFR, related tyrosine kinases, and cell-matrix adhesion receptors.

Acknowledgments

We apologize to those investigators whose contributions were not cited because of space limitations.

References

1. Waterfield, M. D., Scrace, G. T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C. H., Huang, J. S., and Deuel, T. F. Platelet-derived growth factor is structurally related to the putative transforming protein p28sis of simian sarcoma virus. Nature (Lond.), *304:* 35–39, 1983.
2. Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J., and Waterfield, M. D. Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature (Lond.), *307:* 521–527, 1984.
3. Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., *et al*. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells, Nature (Lond.), *309:* 418–425, 1984.
4. Merlino, G. T., Xu, Y. H., Richert, N., Clark, A. J., Ishii, S., Banks-Schlegel, S., and Pastan, I. Elevated epidermal growth factor receptor gene copy number and expression in a squamous carcinoma cell line. J. Clin. Investig., *75:* 1077–1079, 1985.
5. Derynck, R., Goeddel, D. V., Ullrich, A., Gutterman, J. U., Williams, R. D., Bringman, T. S., and Berger, W. H. Synthesis of messenger RNAs for transforming growth factors α and β and the epidermal growth factor receptor by human tumors. Cancer Res., *47:* 707–712, 1987.
6. Costa, S., Stamm, H., Almendral, A., Ludwig, H., Wyss, R., Fabbro, D., Ernst, A., Takahashi, A., and Eppenberger, U. Predictive value of EGF receptor in breast cancer. Lancet, *2:* 1258, 1988.
7. Sainsbury, J. R., Nicholson, S., Angus, B., Farndon, J. R., Malcolm, A. J., and Harris, A. L. Epidermal growth factor receptor status of histological sub-types of breast cancer. Br. J. Cancer, *58:* 458–460, 1988.
8. Kikuchi, A., Amagai, M., Hayakawa, K., Ueda, M., Hirohashi, S., Shimizu, N., and Nishikawa, T. Association of EGF receptor expression with proliferating cells and of ras p21 expression with differentiating cells in various skin tumours. Br. J. Dermatol., *123:* 49–58, 1990.
9. Ibrahim, S. O., Vasstrand, E. N., Liavaag, P. G., Johannessen, A. C., and Lillehaug, J. R. Expression of c-erbB proto-oncogene family members in squamous cell carcinoma of the head and neck. Anticancer Res., *17:* 4539–4546, 1997.
10. Ozawa, S., Ueda, M., Ando, N., Shimizu, N., and Abe, O. Prognostic significance of epidermal growth factor receptor in esophageal squamous cell carcinomas. Cancer (Phila.), *63:* 2169–2173, 1989.
11. Wong, A. J., Ruppert, J. M., Bigner, S. H., Grzeschik, C. H., Humphrey, P. A., Bigner, D. S., and Vogelstein, B. Structural alterations of the epidermal growth factor receptor gene in human gliomas. Proc. Natl. Acad. Sci. USA, *89:* 2965–2969, 1992.
12. Moscatello, D. K., Montgomery, R. B., Sundareshan, P., McDanel, H., Wong, M. Y., and Wong, A. J. Transformational and altered signal transduction by a naturally occurring mutant EGF receptor. Oncogene, *13:* 85–96, 1996.
13. Moscatello, D. K., Holgado-Madruga, M., Emlet, D. R., Montgomery, R. B., and Wong, A. J. Constitutive activation of phosphatidylinositol 3-kinase by a naturally occurring mutant epidermal growth factor receptor. J. Biol. Chem., *273:* 200–206, 1998.
14. Rubin, I., and Yarden, Y. The basic biology of HER2. Ann. Oncol., *12:* S3–S8, 2001.
15. Hynes, N. E., Horsch, K., Olayioye, M. A., and Badache, A. The ErbB receptor tyrosine family as signal integrators. Endocr. Relat. Cancer, *8:* 151–159, 2001.
16. Vardy, D. A., Kari, C., Lazarus, G. S., Jensen, P. J., Zilberstein, A., Plowman, G. D., and Rodeck, U. Induction of autocrine epidermal growth factor receptor ligands in human keratinocytes by insulin/insulin-like growth factor-1. J. Cell. Physiol., *163:* 257–265, 1995.
17. Huang, S. M., Bock, J. M., and Harari, P. M. Epidermal growth factor receptor blockade with C225 modulates proliferation, apoptosis, and radiosensitivity in squamous cell carcinomas of the head and neck. Cancer Res., *59:* 1935–1940, 1999.
18. Ando, Y., and Jensen, P. J. Epidermal growth factor and insulin-like growth factor I enhance keratinocyte migration. J. Investig. Dermatol., *100:* 633–639, 1993.
19. McCawley, L. J., O'Brien, P., and Hudson, L. G. Overexpression of the epidermal growth factor receptor contributes to enhanced ligand-mediated motility in keratinocyte cell lines. Endocrinology, *138:* 121–127, 1997.
20. Chen, C. S., Lavker, R. M., Rodeck, U., Risse, B., and Jensen, P. J. Use of a serum-free epidermal culture model to show deleterious effects of epidermal growth factor on morphogenesis and differentiation. J. Investig. Dermatol., *104:* 107–112, 1995.
21. Peus, D., Hamacher, L., and Pittelkow, M. R. EGF-receptor tyrosine kinase inhibition induces keratinocyte growth arrest and terminal differentiation. J. Investig. Dermatol., *109:* 751–756, 1997.
22. Wang, F., Weaver, V. M., Petersen, O. W., Larabell, C. A., Dedhar, S., Briand, P., Lupu, R., and Bissell, M. J. Reciprocal interactions between beta1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology. Proc. Natl. Acad. Sci. USA, *95:* 14821–14826, 1998.
23. Mendelsohn, J. The epidermal growth factor receptor as a target for cancer therapy. Endocr. Relat. Cancer, *8:* 3–9, 2001.
24. Arteaga, C. L. J. D. H. Tyrosine kinase inhibitors-ZD1839 (Iressa). Curr. Opin. Oncol., *13:* 491–498, 2001.
25. Levitzki, A., and Gazit, A. Tyrosine kinase inhibition: an approach to drug development. Science (Wash. DC), *267:* 1782–1788, 1995.
26. Wu, X., Fan, Z., Masui, H., Rosen, N., and Mendelsohn, J. Apoptosis induced by an anti-epidermal growth factor receptor monoclonal antibody in a human colorectal carcinoma cell line and its delay by insulin. J. Clin. Investig., *95:* 1897–1905, 1995.
27. Rodeck, U., Jost, M., Kari, C., Shih, D-T., Lavker, R. M., Ewert, D. L., and Jensen, P. J. EGF-R dependent regulation of keratinocyte survival. J. Cell Sci., *110:* 113–121, 1997.
28. Jost, M., Gasparro, F. P., Jensen, P. J., and Rodeck, U. Keratinocyte apoptosis induced by UVB radiation and CD95 ligation: differential protection through EGFR activation and Bcl-xL expression. J. Investig. Dermatol., *116:* 860–866, 2001.
29. Frisch, S. M., and Francis, H. Disruption of epithelial cell-matrix interactions induces apoptosis. J. Cell Biol., *124:* 619–626, 1994.
30. Boudreau, N., Werb, Z., and Bissell, M. J. Suppression of apoptosis by basement membrane requires three-dimensional tissue organization and withdrawal from the cell cycle. Proc. Natl. Acad. Sci. USA, *93:* 3509–3513, 1996.
31. Meredith, J. E., Jr., Fazeli, B., and Schwartz, M. A. The extracellular matrix as a cell survival factor. Mol. Biol. Cell, *4:* 953–961, 1993.
32. Le Gall, M., Chambard, J. C., Breittmayer, J. P., Grall, D., Pouyssegur, J., and Van Obberghen-Schilling, E. The p42/p44 MAP kinase pathway prevents apoptosis induced by anchorage and serum removal. Mol. Biol. Cell, *11:* 1103–1112, 2000.
33. Zhu, Z., Sanchez-Sweatman, O., Huang, X., Wiltrout, R., Khokha, R., Zhao, Q., and Gorelik, E. Anoikis and metastatic potential of cloudman S91 melanoma cells. Cancer Res., *61:* 1707–1716, 2001.
34. Frankel, A., Rosen, K., Filmus, J., and Kerbel, R. S. Induction of anoikis and suppression of human ovarian tumor growth *in vivo* by down-regulation of Bcl-X(L). Cancer Res., *61:* 4837–4841, 2001.
35. Rodeck, U., Jost, M., DuHadaway, J., Kari, C., Jensen, P. J., Risse, B., and Ewert, D. L. Regulation of Bcl-xL expression in human keratinocytes by cell-substratum adhesion and the epidermal growth factor receptor. Proc. Natl. Acad. Sci. USA, *94:* 5067–5072, 1997.

36. Gilmore, A. P., Valentijn, A. J., Wang, P., Ranger, A. M., Bundred, N., O'Hare, M. J., Wakeling, A., Korsmeyer, S. J., and Streuli, C. H. Activation of BAD by therapeutic inhibition of epidermal growth factor receptor and transactivation by insulin-like growth factor receptor. J. Biol. Chem., *277:* 27643–27650, 2002.
37. Jost, M., Huggett, T. M., Kari, C., and Rodeck, U. Matrix-independent survival of human keratinocytes through an EGF Receptor/MAPK-kinase-dependent pathway. Mol. Biol. Cell, *12:* 1519–1527, 2001.
38. Valentinis, B., Reiss, K., and Baserga, R. Insulin-like growth factor-I-mediated survival from anoikis: role of cell aggregation and focal adhesion kinase. J. Cell. Physiol., *176:* 648–657, 1998.
39. Zhang, Y. W., Wang, L. M., Jove, R., and Vande Woude, G. F. Requirement of Stat3 signaling for HGF/SF-Met mediated tumorigenesis. Oncogene, *21:* 217–226, 2002.
40. Frisch, S. M., Vuori, K., Ruoslahti, E., and Chan-Hui, P. Y. Control of adhesion-dependent cell survival by focal adhesion kinase. J. Cell Biol., *134:* 793–799, 1996.
41. Delcommenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J., and Dedhar, S. Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. Proc. Natl. Acad. Sci. USA, *95:* 11211–11216, 1998.
42. Attwell, S., Roskelley, C., and Dedhar, S. The integrin-linked kinase (ILK) suppresses anoikis. Oncogene, *19:* 3811–3815, 2000.
43. Wang, S. C., Makino, K., Xia, W., Kim, J. S., Im, S. A., Peng, H., Mok, S. C., Singletary, S. E., and Hung, M. C. DOC-2/hDab-2 inhibits ILK activity and induces anoikis in breast cancer cells through an Akt-independent pathway. Oncogene, *20:* 6960–6964, 2001.
44. White, D. E., Cardiff, R. D., Dedhar, S., and Muller, W. J. Mammary epithelial-specific expression of the integrin-linked kinase (ILK) results in the induction of mammary gland hyperplasias and tumors in transgenic mice. Oncogene, *20:* 7064–7072, 2001.
45. Weaver, V., Lelievre, S., Lakins, J., Chrenek, M., Jones, J., Giancotti, F., Werb, Z., and Bissell, M. $\beta 4$ integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. Cancer Cell, *2:* 205–216, 2002.
46. Debnath, J., Mills, K. R., Collins, N. L., Reginato, M. J., Muthuswamy, S. K., and Brugge, J. S. The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. Cell, *111:* 29–40, 2002.
47. Muthuswamy, S. K., Li, D., Lelievre, S., Bissell, M. J., and Brugge, J. S. ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. Nat. Cell Biol., *3:* 785–792, 2001.
48. Boyer, B., Valles, A. M., and Edme, N. Induction and regulation of epithelial-mesenchymal transitions. Biochem. Pharmacol., *60:* 1091–1099, 2000.
49. Jost, M., Huggett, T. M., Kari, C., Boise, L. H., and Rodeck, U. Epidermal growth factor receptor-dependent control of keratinocyte survival and Bcl-xL expression through a MEK-dependent pathway. J. Biol. Chem., *276:* 6320–6326, 2001.
50. Stoll, S. W., Benedict, M., Mitra, R., Hiniker, A., Elder, J. T., and Nunez, G. EGF receptor signaling inhibits keratinocyte apoptosis: evidence for mediation by Bcl-XL. Oncogene, *16:* 1493–1499, 1998.
51. Schulze, A., Lehmann, K., Jefferies, H. B., McMahon, M., and Downward, J. Analysis of the transcriptional program induced by Raf in epithelial cells. Genes Dev., *15:* 981–994, 2001.
52. Nagane, M., Levitzki, A., Gazit, A., Cavenee, W. K., and Huang, H. J. Drug resistance of human glioblastoma cells conferred by a tumor-specific mutant epidermal growth factor receptor through modulation of Bcl-XL and caspase-3-like proteases. Proc. Natl. Acad. Sci. USA, *95:* 5724–5729, 1998.
53. Mishima, K., Johns, T. G., Luwor, R. B., Scott, A. M., Stockert, E., Jungbluth, A. A., Ji, X. D., Suvarna, P., Voland, J. R., Old, L. J., Huang, H. J., and Cavenee, W. K. Growth suppression of intracranial xenografted glioblastomas overexpressing mutant epidermal growth factor receptors by systemic administration of monoclonal antibody (mAb) 806, a novel monoclonal antibody directed to the receptor. Cancer Res., *61:* 5349–5354, 2001.
54. Fang, X., Yu, S., Eder, A., Mao, M., Bast, R. C., Jr., Boyd, D., and Mills, G. B. Regulation of BAD phosphorylation at serine 112 by the Ras-mitogen-activated protein kinase pathway. Oncogene, *18:* 6635–6640, 1999.
55. Tan, Y., Demeter, M. R., Ruan, H., and Comb, M. J. BAD Ser-155 phosphorylation regulates BAD/Bcl-XL interaction and cell survival. J. Biol. Chem., *275:* 25865–25869, 2000.
56. Zhou, X. M., Liu, Y., Payne, G., Lutz, R. J., and Chittenden, T. Growth factors inactivate the cell death promoter BAD by phosphorylation of its BH3 domain on Ser155. J. Biol. Chem., *275:* 25046–25051, 2000.
57. Fukuzawa, H., Noguchi, Y., Murakami, Y., and Uehara, Y. Mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitors restore anoikis sensitivity in human breast cancer cell lines with a constitutively activated extracellular-regulated kinase (ERK) pathway. Mol. Cancer Ther., *1:* 303–309, 2002.
58. Buettner, R., Mora, L. B., and Jove, R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. Clin. Cancer Res., *8:* 945–954, 2002.
59. Catlett-Falcone, R., Landowski, T. H., Oshiro, M. M., Turkson, J., Levitzki, A., Savino, R., Ciliberto, G., Moscinski, L., Fernandez-Luna, J. L., Nunez, G., Dalton, W. S., and Jove, R. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity, *10:* 105–115, 1999.
60. Grandis, J. R., Drenning, S. D., Chakraborty, A., Zhou, M. Y., Zeng, Q., Pitt, A. S., and Tweardy, D. J. Requirement of Stat3 but not Stat1 activation for epidermal growth factor receptor-mediated cell growth *in vitro*. J. Clin. Investig., *102:* 1385–1392, 1998.
61. Grandis, J. R., Drenning, S. D., Zeng, Q., Watkins, S. C., Melhem, M. F., Endo, S., Johnson, D. E., Huang, L., He, Y. K., and Kim, J. D. Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis *in vivo*. Proc. Natl. Acad. Sci. USA, *97:* 4227–4232, 2000.
62. Okano, J., Gaslightwala, I., Birnbaum, M. J., Rustgi, A. K., and Nakagawa, H. Akt/protein kinase B isoforms are differentially regulated by epidermal growth factor stimulation. J. Biol. Chem., *275:* 30934–30942, 2000.
63. Rytomaa, M., Lehmann, K., and Downward, J. Matrix detachment induces caspase-dependent cytochrome *c* release from mitochondria: inhibition by PKB/Akt but not Raf signalling. Oncogene, *19:* 4461–4468, 2000.
64. Kamalati, T., Jolin, H. E., Fry, M. J., and Crompton, M. R. Expression of the BRK tyrosine kinase in mammary epithelial cells enhances the coupling of EGF signalling to PI 3-kinase and Akt, via erbB3 phosphorylation. Oncogene, *19:* 5471–5476, 2000.
65. Habib, A. A., Chatterjee, S., Park, S. K., Ratan, R. R., Lefebvre, S., and Vartanian, T. The epidermal growth factor receptor engages receptor interacting protein and nuclear factor-κB (NF-κB)-inducing kinase to activate NF-κB. Identification of a novel receptor-tyrosine kinase signalosome. J. Biol. Chem., *276:* 8865–8874, 2001.
66. Bhat-Nakshatri, P., Sweeney, C. J., and Nakshatri, H. Identification of signal transduction pathways involved in constitutive NF-κB activation in breast cancer cells. Oncogene, *21:* 2066–2078, 2002.
67. Chan, T. O., Rodeck, U., Chan, A. M., Kimmelman, A. C., Rittenhouse, S. E., Panayotou, G., and Tsichlis, P. N. Small GTPases and tyrosine kinases coregulate a molecular switch in the phosphoinositide 3-kinase regulatory subunit. Cancer Cell, *1:* 181–191, 2002.
68. Matter, M. L., and Ruoslahti, E. A signaling pathway from the $\alpha 5\beta 1$ and $\alpha(v)\beta 3$ integrins that elevates bcl-2 transcription. J. Biol. Chem., *276:* 27757–27763, 2001.
69. Gilmore, A. P., Metcalfe, A. D., Romer, L. H., and Streuli, C. H. Integrin-mediated survival signals regulate the apoptotic function of Bax through its conformation and subcellular localization. J. Cell Biol., *149:* 431–446, 2000.
70. Kantak, S. S., and Kramer, R. H. E-cadherin regulates anchorage-independent growth and survival in oral squamous cell carcinoma cells. J. Biol. Chem., *273:* 16953–16961, 1998.

DOI:10.1111/j.1600-0625.2007.00563.x
www.blackwellpublishing.com/EXD

Anchorless keratinocyte survival: an emerging pathogenic mechanism for squamous cell carcinoma in recessive dystrophic epidermolysis bullosa

Ulrich Rodeck, Andrzej Fertala and Jouni Uitto

Department of Dermatology and Cutaneous Biology, Jefferson Medical College, Thomas Jefferson University, and Jefferson Institute of Molecular Medicine, Philadelphia, PA, USA

Correspondence: Ulrich Rodeck, Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, 233 S. 10th Street, BLSB409, Philadelphia, PA 19107, USA, Tel.: 215-503-5622, Fax: 215-503-5622, e-mail: ulrich.rodeck@mail.jci.tju.edu

[1]Previously known as laminin 5; for the revised nomenclature on laminins, see Aumailley et al. Matrix Biol 2005: 24: 326–332.

Accepted for publication 9 March 2007

Abstract: Squamous cell carcinomas in patients sufering from recessive dystrophic epidermolysis bullosa are highly invassive and frequently metastatic. Expression of a collagen VII fragement (NC1) has been described as a prerequisite for the development of this tumor form. This commentary focuses on potential molecular mechanisms by which expression of the NC1 fragment may augment anchorage-independent growth and survival of malignant keratinocytes.

Key words: carcinoma – collagen type VII – Epidermolysis bullosa dystrophica – signal transduction – squamous cell

Please cite this paper as: Anchorless keratinocyte survival: an emerging pathogenic mechanism for squamous cell carcinoma in recessive dystrophic epidermolysis bullosa. Experimental Dermatology 2007; 16: 465–467.

Epidermolysis bullosa (EB), a group of mechanobullous blistering disorders, is characterized by extensive clinical heterogeneity. Certain subtypes, such as the recessive dystrophic variants (RDEB), are associated with considerable morbidity and premature demise of the affected individuals within the first four decades of life. Although recent advances in the clinical management of RDEB have significantly extended the lifespan of the affected individuals, RDEB patients increasingly magnifest a major life-threatening complication related to the development of squamous cell carcinomas (SCCs). RDEB-associated SCCs manifest early in life, and are distinguished by a particularly aggressive clinical course. Very high rates of metastatic spread have been observed, rendering skin cancer to be a major cause of death in RDEB patients (1). Despite the life-threatening nature of SCC in RDEB patients, reports on the pathogenesis of RDEB-associated SCCs are scarce. For example, Arbiser et al. (2) described mutations in the p53 tumor suppressor gene in three out of eight RDEB SCC samples. Consistent with compromised p53 function (3,4), RDEB-associated SCC also expresses reduced levels of the insulin-like growth factor binding protein IGFBP-3 (5). In addition, both RDEB-associated and sporadic SCC exhibit enhanced expression of the transmembrane glycoprotein MUC1 (6). Yet, it remains largely unknown whether RDEB-associated tumors represent a distinct entity or share pathways to malignancy with SCCs in the general non-RDEB population. This lack of information has precluded the rational administration of targeted therapies increasingly considered in the management of SCCs.

A recent study has highlighted a potentially interesting feature of RDEB-associated SCC related to the expression of a collagen VII fragment. Collagen VII is the predominant, if not the exclusive, component of anchoring fibrils, attachment structures extending from the lamina densa of the dermo-epidermal basement membrane to the upper papillary dermis and providing stability to the cutaneous basement membrane zone (BMZ) (Fig. 1). In a substantial number of patients with RDEB the collagen VII gene, COL7A1, harbors nonsense mutations, giving rise to premature termination codons, and consistent with expression of truncated collagen VII (7). In fact, expression of a collagen VII fragment that corresponds to the N-terminal non-collagenous domain (NC1) has recently been shown to be necessary for tumorigenic conversion of keratinocytes cultured from RDEB patients xenotransplanted to immunodeficient mice (8). Conversely, RDEB keratinocytes which did not express the NC1 domain did not develop SCCs.

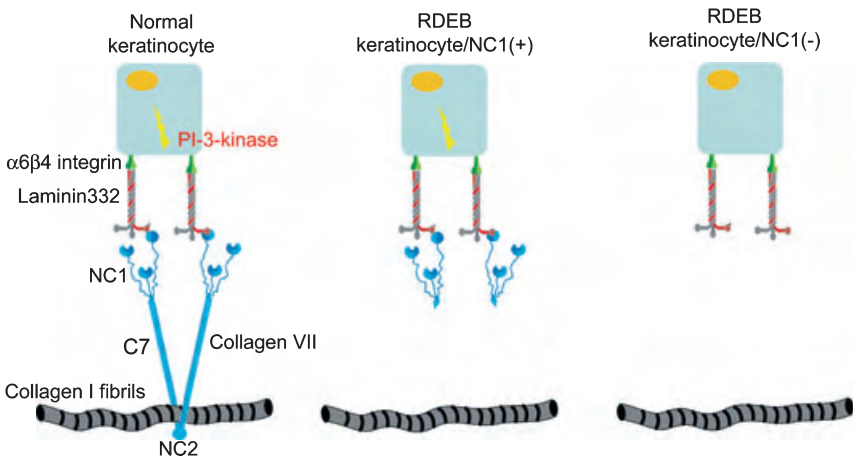

Figure 1. Schematic representation of 'anchorless' activation of $\alpha 6\beta 4$ integrin-mediated signal transduction in RDEB keratinocytes. In normal skin, collagen VII is firmly anchored to the basement membrane zone (BMZ)/dermis interface through interactions with other components of the extracellular matrix, such as collagen I. Thus, activation of $\alpha 6\beta 4$ integrin is restricted to the appropriate tissue compartment within the epidermis, i.e. the basal keratinocytes. In contrast, expression of N-terminally truncated collagen VII lacking the C7 and NC2 domains, may enable $\alpha 6\beta 4$ integrin-dependent signal transduction in RDEB keratinocytes which are not firmly anchored in the BMZ, potentially supporting inappropriate cell survival during invasion and metastasis. In the case of complete absence of NC1 expression, activation of $\alpha 6\beta 4$ integrin-dependent signal transduction will not occur.

The notion that NC1 expression is required for SCC development in RDEB patients was recently challenged by the isolation of keratinocytes from two RDEB patients with SCC that lack procollagen VII expression (9). It is also worth noting that NC1-dependent tumor-formation has as yet only been described in keratinocytes that were immortalized by the coexpression of Ha-RasV12 and mutant IκBα to inhibit NF-κB activity. Ha-Ras mutations are relatively infrequent in sporadic SCC (10–14) and, at least in fibroblasts, Ha-Ras itself has significant effects on the production of extracellular matrix components, including fibronectin and collagen I (15). This complicates the interpretation of results in Ha-Ras-V12 expressing keratinocytes, as they relate to extracellular matrix composition and tumor-formation. Thus, it will be important to resolve whether NC1 expression is required for tumorigenesis in models of RDEB SCC other than the Ha-RasV12/IκBα model.

Ortiz-Urda and colleagues (8,16) also proposed a molecular mechanism by which NC1 could participate in tumor development based on studies using antibodies reactive with NC1 sequences and predicted to disrupt the interaction of NC1 with laminin 332[1]. These antibodies inhibited tumorigenicity of Ha-RasV12-transformed NC1-expressing RDEB keratinocytes and inhibited PI-3-kinase activity in these cells. PI-3-kinase activation has been previously shown to provide robust survival signals to multiple epithelial cell types (17,18), including keratinocytes (19). Furthermore, in keratinocytes, integrin-mediated matrix adhesion significantly contributes to PI-3-kinase/Akt activation (19,20). Incidentally, aberrant high-level expression of laminin 332 has also been shown to induce anchorage-independent survival of malignant mammary epithelial cells dependent on $\alpha 6\beta 4$ integrin expression and Rac/NF-κB signaling (21). Collectively, these results suggest the intriguing possibility that the NC1/laminin 332 interaction serves to trigger integrin-mediated signals essential for the survival of transformed keratinocytes in the anchorage-independent state. Implicitly, these results also suggest that the collagenous (C7) and C-terminal (NC2) domains of procollagen VII are not required for $\alpha 6\beta 4$ integrin-mediated survival signaling. This provokes the question whether other means of blocking NC1 expression or function, for example antisense approaches, similarly disrupt tumorigenicity and signal transduction in NC1-expressing keratinocytes.

The extensive and recurrent blistering in RDEB patients attests to the fact that the C7/NC2 domains are essential for anchoring keratinocytes to the BMZ. C7/NC2-dependent BMZ attachment may also serve a critical role in preventing the dissemination of keratinocytes to inappropriate sites, as observed during the metastatic process. Thus, it is possible that the convergence of two mechanisms contributes to the clinical aggressiveness of SCCs in RDEB patients expressing NC1 (Fig. 1). Whereas loss of C7/NC2-dependent cell anchorage enables keratinocyte dissemination, retention of the NC1 domain enables signal transduction in support of cell survival during transit to metastatic sites. Finally, RDEB patients do not develop SCCs immediately after birth but in later life. Thus, oncogenic mechanisms other than NC1-dependent events are likely to contribute to the clinically aggressive, metastatic phenotype of RDEB-associated SCCs and potentially related to the extensive scarring associ-

ated with RDEB. Whereas the precise nature of cooperating oncogenic events in RDEB-associated SCC is unknown, it would be worthwhile to investigate whether such mechanisms affect molecular pathways in sporadic SCCs as well.

References

1 Fine J D, Johnson L B, Suchindran C *et al*. Cancer and inherited epidermolysis bullosa: lifetable analyses of the national epidermolysis bullosa registry study population. In: Fine J D, Bauer E A, McGuire J, Moshell A, eds. Epidermolysis Bullosa. Baltimore: The Johns Hopkins University Press, 1999: 175–192.

2 Arbiser J L, Fan C Y, Su X *et al*. Involvement of p53 and p16 tumor suppressor genes in recessive dystrophic epidermolysis bullosa-associated squamous cell carcinoma. J Invest Dermatol 2004: **123**: 788–790.

3 Ryan K M, Vousden K H. Characterization of structural p53 mutants which show selective defects in apoptosis but not cell cycle arrest. Mol Cell Biol 1998: **18**: 3692–3698.

4 Hollowood A D, Lai T, Perks C M, Newcomb P V, Alderson D, Holly J M. IGFBP-3 prolongs the p53 response and enhances apoptosis following UV irradiation. Int J Cancer 2000: **88**: 336–341.

5 Mallipeddi R, Wessagowit V, South A P *et al*. Reduced expression of insulin-like growth factor-binding protein-3 (IGFBP-3) in squamous cell carcinoma complicating recessive dystrophic epidermolysis bullosa. J Invest Dermatol 2004: **122**: 1302–1309.

6 Cooper H L, Cook I S, Theaker J M *et al*. Expression and glycosylation of MUC1 in epidermolysis bullosa-associated and sporadic cutaneous squamous cell carcinomas. British J Dermatol 2004: **151**: 540–545.

7 Varki R, Sadowski S, Uitto J, Pfendner E. Epidermolysis bullosa. II. Type VII collagen mutations and phenotype/genotype correlations in the dystrophic subtypes. J Med Genet, Epub ahead of print 2006: **44**: 181–192.

8 Ortiz-Urda S, Garcia J, Green C L *et al*. Type VII collagen is required for Ras-driven human epidermal tumorigenesis. Science 2005: **307**: 1773–1776.

9 Pourreyron C, Jones C, Volz A *et al*. Patients with recessive dystrophic epidermolysis bullosa develop squamous cell carcinoma regardless of type VII collagen NC1 domain expression. J Invest Dermatol 2006: **126** (Suppl. 3): s32.

10 Campbell C, Quinn A G, Rees J L. Codon 12 Harvey-ras mutations are rare events in non-melanoma human skin cancer. British J Dermatol 1993: **128**: 111–114.

11 Pierceall W E, Goldberg L H, Tainsky M A, Mukhopadhyay T, Ananthaswamy H N. Ras gene mutation and amplification in human nonmelanoma skin cancers. Mol Carcinog 1991: **4**: 196–202.

12 Rumsby G, Carter R L, Gusterson B A. Low incidence of ras oncogene activation in human squamous cell carcinomas. Br J Cancer 1990: **61**: 365–368.

13 van der Schroeff J G, Evers L M, Boot A J, Bos J L. Ras oncogene mutations in basal cell carcinomas and squamous cell carcinomas of human skin. J Invest Dermatol 1990: **94**: 423–425.

14 Clark L J, Edington K, Swan I R *et al* The absence of Harvey ras mutations during development and progression of squamous cell carcinomas of the head and neck. Br J Cancer 1993: **68**: 617–620.

15 Martinez-Salgado C, Fuentes-Calvo I, Garcia-Cenador B, Santos E, Lopez-Novoa J M. Involvement of H- and N-Ras isoforms in transforming growth factor-beta1-induced proliferation and in collagen and fibronectin synthesis. Exp Cell Res 2006: **312**: 2093–2106.

16 Sakai N, Waterman E A, Nguyen N E *et al*. Laminin 5 beta 3 chain promotes epidermal carcinogenesis through type VII collagen binding and PI3-kinase activation. J Invest Dermatol 2006: **126**: 24.

17 Khwaja A, Rodriguez-Viciana P, Wennstrom S, Warne P H, Downward J. Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. EMBO J 1997: **16**: 2783–2793.

18 Kennedy S G, Wagner A J, Conzen S D *et al*. The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. Genes Dev. 1997: **11**: 701–713.

19 Chan T O, Rodeck U, Chan A M *et al*. Small GTPases and tyrosine kinases coregulate a molecular switch in the phosphoinositide 3-kinase regulatory subunit. Cancer Cell 2002: **1**: 181–191.

20 Nguyen B P, Gil S G, Carter W G. Deposition of laminin 5 by keratinocytes regulates integrin adhesion and signaling. J Biol Chem 2000: **275**: 31896–31907.

21 Zahir N, Lakins J N, Russell A *et al*. Autocrine laminin-5 ligates alpha6beta4 integrin and activates RAC and NFkappaB to mediate anchorage-independent survival of mammary tumors. J Cell Biol 2003: **163**: 1397–1407.

VOLUME 23 · NUMBER 24 · AUGUST 20 2005

JOURNAL OF CLINICAL ONCOLOGY

EDITORIAL

Predicting the Future From Trials of the Past: Epidermal Growth Factor Receptor Expression and Outcome of Fractionated Radiation Therapy Trials

Adam P. Dicker and Ulrich Rodeck, *Departments of Radiation Oncology and Dermatology, Thomas Jefferson University, Philadelphia, PA*

The epidermal growth factor receptor (EGFR; HER 1; ErbB1) has been at the center of an explosion of translational research over the past 20 years. The EGFR is expressed or overexpressed in a large number of malignancies, including non–small-cell lung (NSCLC), head and neck, esophageal, gastric, colorectal, breast, prostate, bladder, renal, pancreatic, and ovarian cancers.[1,2] EGFR expression is often found in association with increased expression of its ligands, most notably epidermal growth factor (EGF), tumor growth factor-alpha, or amphiregulin.[3] EGFR activation and signaling affects many aspects of cell biology relevant to malignant transformation, including cell cycle progression, migration and invasion, differentiation, and cell survival. The antiapoptotic effects of EGFR activation may be particularly relevant to the assessment of treatment outcomes in patients afflicted with EGFR expressing tumors have been ascribed to regulation of Bcl-2 family members.[4-10]

In this issue, Bentzen et al[11] examine EGFR expression status in 304 patients with available pretreatment tumor biopsy material among 918 patients randomized to Continuous Hyperfractionated Accelerated Radiotherapy (CHART) versus conventionally fractionated radiotherapy. The EGFR index was defined as the proportion of tumor cells with EGFR membrane staining. Significant benefit in locoregional tumor control from CHART was seen in patients with head and neck squamous cell carcinoma with high EGFR expression ($2P = .010$) but not in patients with low EGFR expression ($2P = .85$). By contrast, EGFR expression status was not linked to patient survival or rate of distant metastases.

Bentzen et al[11] used immunohistochemical analysis to assess EGFR expression levels, which raises some technical concerns. First, manual methods of immunohistochemical scoring of antigen expression as used in this report are fraught with variability. This variability may explain the lack of association of EGFR expression with other biomarkers evaluated in this study (Ki-67 index, Ki-67 pattern, p53 index, p53 intensity, bcl-2 expression, or cyclin D1 index). Another concern centers on the retrospective analysis of archived tissue stored for different periods of time. The CHART head and neck phase III trial accrued patients over 5 years from March 1990 to April 1995. During this extended period, oxidation of antigens/DNA/RNA may have occurred. A recent editorial in the *Journal of Clinical Oncology* by Meropol[12] refers to work by Atkins et al[13] in support of the notion that extended storage of tissue sections over time affects the sensitivity of EGFR immunostaining. However, despite these concerns, the study by Bentzen et al[11] provides an interesting new perspective on intratumoral EGFR expression as a variable with obvious relevance to the design of fractionated radiotherapy.

Several reports have illuminated molecular mechanisms leading to enhanced expression of the EGFR in epithelial malignancies. In addition to gene amplification, sequence analysis has revealed polymorphisms of intron 1 of the *EGFR* gene characterized by (CA)n dinucleotide repeats of different lengths that regulate transcription rates of the EGFR in cell lines and in tumor tissues in patients.[14-17] The length of the (CA)n dinucleotide repeat tract is inversely correlated with the transcriptional activity of the *EGFR* gene.

Further investigation of EGFR sequences expressed by tumor cells has also revealed alterations that primarily affect the activation state of the receptor rather than the expression levels. For example, in patients with colorectal cancer, a polymorphic variant of the *EGFR* gene (HER-1 R497K) has

been identified that is associated with higher receptor kinase activity.[18] Interestingly, expression of the constitutively active HER-1 R497K variant is associated with pelvic tumor recurrences. It remains to be investigated whether this EGFR variant is expressed at normal or increased levels in tumor cells.

Other studies revealed somatic mutations representing small deletions, mutations, or missense point mutations in the tyrosine kinase domain of *EGFR* gene, which greatly affect sensitivity of these tumors to EGFR inhibitors and ionizing radiation.[19-22] These mutations result not only in constitutive activity of the EGFR and enhanced long-term activation by its ligands but also in exceptional sensitivity toward EGFR inhibitors such as gefinitib (IRESSA).[23-27] Although these tyrosine kinase domain mutations are best characterized in NSCLC they may be relevant for other cancers, as described recently for colorectal tumors[28] and head and neck cancer.[29] Collectively, these results raise the important issue of whether determining EGFR expression levels alone should guide future radiotherapy trials in patients afflicted with head and neck squamous cell carcinomas. The experience with gefinitib clearly suggests that screens for EGFR mutations should complement assessment of EGFR expression status to identify select groups of patients that may benefit from different radiation therapy modalities.

Two major developments since the CHART trial are the use of chemotherapy and the EGFR antagonistic monoclonal antibody cetuximab in head and neck malignancies. Several trials have shown the benefit of chemotherapy delivered concurrently with radiotherapy, which primarily improves the local or locoregional effect(s) of radiotherapy.[30] Furthermore, multiple reports support the view that EGFR blockade radiosensitizes carcinoma cells in both in vitro and in vivo settings.[31-38] The international phase III trial report by Bonner et al[39] demonstrated that addition of an anti-EGFR antibody to radiation yielded an improvement in locoregional tumor control and overall survival without increasing mucositis and dysphagia compared with radiation alone. These studies raise the issue of whether EGFR expression and mutational status can be used to identify patients with head and neck cancer who would benefit from different radiation treatment modalities perhaps in combination with EGFR inhibitors.

In summary, the report by Bentzen et al[11] suggests that more aggressive radiation therapy may be useful to treat biologically aggressive head and neck squamous cell carcinomas. This work underscores the importance of retrospective biomarker research to guide hypothesis-driven prospective clinical trials. It is likely that future studies will include analysis of the mutational or activation status of the EGFR in tumor tissues. This will provide a sound basis for future research efforts aimed at integrating cutting-edge therapies in the treatment of head and neck cancer.

Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

REFERENCES

1. Bunn PA Jr, Franklin W: Epidermal growth factor receptor expression, signal pathway, and inhibitors in non-small cell lung cancer. Semin Oncol 29:38-44, 2002

2. Salomon DS, Brandt R, Ciardiello F, et al: Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol 19:183-232, 1995

3. Baselga J: Why the epidermal growth factor receptor? The rationale for cancer therapy. Oncologist 7:2-8, 2002 (suppl 4)

4. Bill HM, Knudsen B, Moores SL, et al: Epidermal growth factor receptor-dependent regulation of integrin-mediated signaling and cell cycle entry in epithelial cells. Mol Cell Biol 24:8586-8599, 2004

5. Reginato MJ, Mills KR, Paulus JK, et al: Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. Nat Cell Biol 5:733-740, 2003

6. Jost M, Class R, Kari C, et al: A central role of Bcl-X(L) in the regulation of keratinocyte survival by autocrine EGFR ligands. J Invest Dermatol 112:443-449, 1999

7. Jost M, Huggett TM, Kari C, et al: Matrix-independent survival of human keratinocytes through an EGF receptor/MAPK-kinase-dependent pathway. Mol Biol Cell 12:1519-1527, 2001

8. Jost M, Huggett TM, Kari C, et al: Epidermal growth factor receptor-dependent control of keratinocyte survival and Bcl-xL expression through a MEK-dependent pathway. J Biol Chem 276:6320-6326, 2001

9. Mahoney MG, Simpson A, Jost M, et al: Metastasis-associated protein (MTA)1 enhances migration, invasion, and anchorage-independent survival of immortalized human keratinocytes. Oncogene 21:2161-2170, 2002

10. Schulze A, Lehmann K, Jefferies HB, et al: Analysis of the transcriptional program induced by Raf in epithelial cells. Genes Dev 15:981-994, 2001

11. Bentzen SM, Atasoy BM, Daley FM, et al: Epidermal growth factor receptor expression in pre-treatment biopsies from head and neck squamous cell carcinoma as a predictive factor for a benefit from accelerated radiation therapy in a randomized controlled trial. J Clin Oncol 23:5560-5567, 2005

12. Meropol NJ: Epidermal growth factor receptor inhibitors in colorectal cancer: It's time to get back on target. J Clin Oncol 23:1791-1793, 2005

13. Atkins D, Reiffen KA, Tegtmeier CL, et al: Immunohistochemical detection of EGFR in paraffin-embedded tumor tissues: Variation in staining intensity due to choice of fixative and storage time of tissue sections. J Histochem Cytochem 52:893-901, 2004

14. Gebhardt F, Zanker KS, Brandt B: Differential expression of alternatively spliced c-erbB-2 mRNA in primary tumors, lymph node metastases, and bone marrow micrometastases from breast cancer patients. Biochem Biophys Res Commun 247:319-323, 1998

15. Gebhardt F, Zanker KS, Brandt B: Modulation of epidermal growth factor receptor gene transcription by a polymorphic dinucleotide repeat in intron 1. J Biol Chem 274:13176-13180, 1999

16. Gebhardt F, Burger H, Brandt B: Modulation of EGFR gene transcription by a polymorphic repetitive sequence–a link between genetics and epigenetics. Int J Biol Markers 15:105-110, 2000

17. Buerger H, Gebhardt F, Schmidt H, et al: Length and loss of heterozygosity of an intron 1 polymorphic sequence of egfr is related to cytogenetic alterations and epithelial growth factor receptor expression. Cancer Res 60:854-857, 2000

18. Zhang W, Park DJ, Lu B, et al: Epidermal growth factor receptor gene polymorphisms predict pelvic recurrence in patients with rectal cancer treated with chemoradiation. Clin Cancer Res 11:600-605, 2005

19. Dowell JE, Minna JD: Chasing mutations in the epidermal growth factor in lung cancer. N Engl J Med 352:830-832, 2005

20. Sellers WR, Meyerson M: EGFR gene mutations: A call for global x global views of cancer. J Natl Cancer Inst 97:326-328, 2005

21. Paez JG, Janne PA, Lee JC, et al: EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. Science 304:1497-1500, 2004

22. Sorscher SM, Rich JN, Rasheed BK, et al: EGFR mutations and sensitivity to gefitinib. N Engl J Med 351:1260-1261, 2004

23. Kosaka T, Yatabe Y, Endoh H, et al: Mutations of the epidermal growth factor receptor gene in lung cancer: Biological and clinical implications. Cancer Res 64:8919-8923, 2004

24. Marchetti A, Martella C, Felicioni L, et al: EGFR mutations in non-small-cell lung cancer: Analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. J Clin Oncol 23:857-865, 2005

25. Shigematsu H, Lin L, Takahashi T, et al: Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. J Natl Cancer Inst 97:339-346, 2005

26. Han SW, Kim TY, Hwang PG, et al: Predictive and prognostic impact of epidermal growth factor receptor mutation in non-small-cell lung cancer patients treated with gefitinib. J Clin Oncol 23:2493-2501, 2005

27. Pao W, Miller V, Zakowski M, et al: EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A 101:13306-13311, 2004

28. Nagahara H, Mimori K, Ohta M, et al: Somatic mutations of epidermal growth factor receptor in colorectal carcinoma. Clin Cancer Res 11:1368-1371, 2005

29. Lee J, Soung Y, Kim S, et al: Somatic mutations of EGFR gene in squamous cell carcinoma of the head and neck. Clin Cancer Res 11:2879-2882, 2005

30. Cooper JS, Ang KK: Concomitant chemotherapy and radiation therapy certainly improves local control. Int J Radiat Oncol Biol Phys 61:7-9, 2005

31. Raben D, Bianco C, Helfrich B, et al: Interference with EGFR signaling: Paradigm for improving radiation response in cancer treatment. Expert Rev Anticancer Ther 2:461-471, 2002

32. Bianco C, Tortora G, Bianco R, et al: Enhancement of antitumor activity of ionizing radiation by combined treatment with the selective epidermal growth factor receptor-tyrosine kinase inhibitor ZD1839 (Iressa). Clin Cancer Res 8:3250-3258, 2002

33. Buchsbaum DJ, Bonner JA, Grizzle WE, et al: Treatment of pancreatic cancer xenografts with Erbitux (IMC-C225) anti-EGFR antibody, gemcitabine, and radiation. Int J Radiat Oncol Biol Phys 54:1180-1193, 2002

34. Huang SM, Harari PM: Modulation of radiation response after epidermal growth factor receptor blockade in squamous cell carcinomas: Inhibition of damage repair, cell cycle kinetics, and tumor angiogenesis. Clin Cancer Res 6:2166-2174, 2000

35. Harari PM, Huang SM: Modulation of molecular targets to enhance radiation. Clin Cancer Res 6:323-325, 2000

36. Reardon DB, Contessa JN, Mikkelsen RB, et al: Dominant negative EGFR-CD533 and inhibition of MAPK modify JNK1 activation and enhance radiation toxicity of human mammary carcinoma cells. Oncogene 18:4756-4766, 1999

37. Nakata E, Hunter N, Mason K, et al: C225 antiepidermal growth factor receptor antibody enhances the efficacy of docetaxel chemoradiotherapy. Int J Radiat Oncol Biol Phys 59:1163-1173, 2004

38. Milas L, Mason K, Hunter N, et al: In vivo enhancement of tumor radioresponse by C225 antiepidermal growth factor receptor antibody. Clin Cancer Res 6:701-708, 2000

39. Bonner J, Giralt J, Harari P, et al: Cetuximab prolongs survival in patients with locoregionally advanced squamous cell carcinoma of head and neck: A phase III study of high dose radiation therapy with or without cetuximab. Proc Am Soc Clin Oncol 23:489, 2004 (abstr 5507)