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EGF-R dependent regulation of keratinocyte survival

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SUMMARY

Tissue organization and maintenance within multicellular organisms is in part dependent on the ability of cells to undergo programmed cell death or apoptosis. Conversely, disruption of cell death pathways often is associated with tumor development. At present, the molecular control of apoptosis in epithelial cells is poorly understood. Here we describe evidence linking epidermal growth factor-receptor (EGF-R) activation to survival of normal human keratinocytes in culture. Inhibition of EGF-R activation by an anti-EGF-R antagonistic monoclonal antibody (mAb 425), followed by detachment of keratinocytes from the substratum, induced extensive death with several features of apoptosis in keratinocyte cultures. Other, non-epithelial normal human cells including melanocytes and fibroblasts, did not show this effect. Similar to EGF-R blockade by mAb 425, inhibition of the EGF-R tyrosine kinase activity using tyrphostin AG1478 resulted in lack of attachment and extensive cell death upon passaging. Attachment to keratinocyte-derived ECM partially rescued mAb 425-treated keratinocytes from cell death, indicating that adhesion-dependent and EGF-R-dependent signal transduction pathways serve partially overlapping but not redundant roles in supporting keratinocyte survival.

Key words: EGF receptor, Programmed cell death, Keratinocyte, Integrin

INTRODUCTION

Much evidence supports the hypothesis that epidermal growth factor receptor (EGF-R) activation is central to growth regulation in normal epidermal keratinocytes. In at least one culture model, exogenous EGF is necessary for long-term propagation of these cells (Rheinwald and Green, 1977). More recently, autocrine ligands of the EGF-R have been implicated in regulation of keratinocyte growth (Vardy et al., 1995). At least 3 ligands of the EGF-R, including transforming growth factor-α (TGF-α; Coffey et al., 1987), amphiregulin (AR; Cook et al., 1991a), and heparin-binding-EGF (Hashimoto et al., 1994) are synthesized by normal keratinocytes both in vitro and in vivo. Furthermore, the EGF-R is most strongly expressed in the basal layer of epidermis (Nanney et al., 1984, 1990), in which the proliferating keratinocytes reside.

In recent years, some growth factors/cytokines have been assigned roles not only in growth control but also in regulation of cell survival (for review see White, 1996). For example, survival of fibroblasts has been shown to depend on IGF-1 (Sell et al., 1995) and that of hemopoietic stem cells on IL-3 (Vaux et al., 1988); removal of these growth factors or blockade of their receptors leads to induction of apoptosis in the respective target cells. In support of the idea that growth factors also control certain aspects of keratinocyte terminal differentiation and death, a recent report showed that keratinocyte growth factor (KGF) can delay keratinocyte differentiation and nuclear fragmentation induced by confluence and/or suspension culture (Hines and Allen-Hoffman, 1996). That the EGF-R may similarly regulate apoptosis is suggested by studies with the hemopoietic progenitor cell line 32D. These cells depend on interleukin-3 (IL-3) for survival, as demonstrated by the finding that they undergo apoptosis upon IL-3 withdrawal (Greenberger et al., 1983). When 32D cells were transfected with an expression vector for the EGF-R, addition of EGF to the culture medium rescued cell death induced by IL-3 removal (Pierce et al., 1988). This result indicates that EGF-R-dependent signal transduction pathways can contribute to survival in hemopoietic cells that do not normally express the EGF-R.

In the present study, we tested the hypothesis that the EGF-R can contribute to survival of normal keratinocytes. We utilized early passage cultures of human neonatal keratinocytes in which EGF-R activation was inhibited with an antagonistic anti-EGF-R monoclonal antibody (mAb). The results reported herein indicate that EGF-R activation and integrin-mediated substrate adhesion cooperate to promote survival of normal keratinocytes.

MATERIALS AND METHODS

Cell culture and reagents

Human neonatal foreskin keratinocyte cultures were initiated and propagated in MCDB153 complete medium, as previously described (McNeill and Jensen, 1990). Complete MCDB153 (Sigma, St Louis, MO) contained 30 μM Ca^{2+} and was supplemented with amino acids (histidine 2.4×10^{-4} M, isoleucine 7.5×10^{-4} M, methionine 9.0×10^{-5}

M, phenylalanine 9.0×10^{-5} M, tryptophan 4.5×10^{-5} M, tyrosine 7.5×10^{-5} M; all from Sigma); 10^{-4} M ethanolamine; 10^{-4} M phosphorylethanolamine (Sigma); hydrocortisone (5×10^{-7} M; Sigma); EGF (10 ng/ml, Collaborative Research, Bedford, MA); insulin (5 μg/ml, Sigma); and bovine pituitary extract (prepared from pituitaries from Pel-Freez, Rogers, AR) at 140 μg/ml. All growth assays were performed using MCDB153 complete medium free of exogenous EGF. Human recombinant keratinocyte growth factor (KGF) was purchased from Sigma.

Antigen specificity and EGF-R antagonistic properties of mAb 425, which recognizes a protein epitope on the human EGF-R, have been described in detail (Murthy et al., 1987, 1990; Rodeck et al., 1987, 1990). mAb 15-6A, which is directed to a carbohydrate epitope, binds to but does not interfere with the activity of the EGF-R (Basu et al., 1987). Antibodies were purified by Protein A-Sepharose chromatography, dialyzed extensively against phosphate-buffered saline (PBS), and sterile filtered. In previous studies we have shown that the mAb 425 preparation used here is non-toxic, as high concentrations of EGF neutralize the effect of mAb 425 on keratinocyte proliferation (Vardy et al., 1995). The IGF-1-R antagonistic mAb IR-3 has been described previously (Jacobs et al., 1986). Integrin antibodies for immunoprecipitation analyses were a kind gift from Dr C. Buck (The Wistar Institute, Philadelphia). For flow cytometric analysis of integrin expression the following monoclonal antibodies were used: anti-β1 (rat) provided by Dr C. Damsky (University of California, San Francisco, CA); anti-α6 (rat) provided by Dr M. Wheelock (University of Toledo, Toledo, OH); anti-α2 (mouse; Oncogene Science, Uniondale, NY); anti-α3 (mouse; Oncogene Science); anti-α5 (mouse; Oncogene Science). Tyrphostin AG 1478 was generously provided by Dr A. Levitzki (Hebrew University of Jerusalem, Israel).

Preparation of keratinocyte-derived extracellular matrix

To generate keratinocyte-derived extracellular matrix, confluent, fourth passage keratinocyte cultures were detached from 6-well plates by treatment for 5-10 minutes with 0.05% trypsin and 1 mM EDTA in PBS. The matrix-coated wells were then washed in PBS, treated with 0.1 mg/ml soybean trypsin inhibitor, washed again, blocked overnight at 4°C with 1 mg/ml BSA (fraction V from Sigma, #A8412) in PBS, and stored in sterile PBS at 4°C until use. Xia et al. (1996) have shown that laminin 5 (or epiligrin) is the major adhesive ligand on plates so treated. Alternatively, in some experiments, keratinocytes were removed from wells by repeated freeze/thawing using an ethanol/dry ice slurry. Similar data were obtained using plates prepared by either method.

Apoptosis assays

DNA breaks were detected by end-labeling using a previously published method (Gorczyca et al., 1993) with minor modifications. Briefly, for flow cytometry 10^6 cells were fixed for 10 minutes in 1% paraformaldehyde in PBS, followed by 10 minutes fixation in 80% ethanol. After washing in PBS supplemented with 1% bovine serum albumin (PBS/BSA), cells were resuspended in 50 μl of terminal-deoxy-transferase (TdT) reaction mixture (200 mM potassium cacodylate, 25 mM Tris-HCl, pH 6.6, 250 μg/ml BSA, 1 mM $CoCl_2$, 3 nM dATP, 0.3 nM FITC-12-dUTP (Boehringer Mannheim; Indianapolis, IN), and 20 units TdT (Boehringer Mannheim) and incubated for 1 hour at 37°C. Cells were washed once in PBS/BSA, followed by flow cytometric analysis using an EPICS XL flow cytometer (Coulter Counter, Hialeah, FL). For in situ end-labeling cells were trypsinized; and 10^5 cells were cytocentrifuged onto microscope slides, fixed in 1% paraformaldehyde in PBS for 20 minutes, air-dried, and incubated with the same TdT reaction mixture used for flow cytometric analysis except that biotin-11-dUTP (Enzo Diagnostics Inc., Farmingdale, NY) was substituted for FITC-12-dUTP. The slides were then incubated with horseradish peroxidase-conjugated streptavidin for 20 minutes at room temperature. After washing in PBS slides were incubated with 3′3′diaminobenzidine substrate for 5 minutes. Slides were counterstained with light green.

Intracellular DNA was quantitatively measured by incubating cells, which had been fixed in 80% ethanol for a minimum of 20 minutes, with PBS containing 20 μg/ml propidium iodide and 6 μg/ml RNAse A, followed by flow cytometric analysis using an EPICS XL flow cytometer.

Visual assessment of DNA condensation and nuclear fragmentation was made by light microscopic examination of acridine orange (AO) stained cells and electron microscopic examination of cytospin preparations. For AO staining, 10^5 cells in 10 μl PBS were mixed with 2 μl of AO (50 μg/ml) on a microscope slide, coverslipped, and examined using a Leitz Orthoplan fluorescence microscope with epi-illumination. For EM, cells were treated with Karnovski's fixative (containing 2% paraformaldehyde and 2.5% glutaraldehyde; Polysciences, Warrington, PA), dehydrated and embedded in Epon 812 (Polysciences), as previously described (Lavker and Sun, 1983). Thin sections (400 Å) were cut, stained with uranyl acetate, and examined with an Hitachi H7000 electron microscope.

Growth assays

Effects of various treatments on keratinocyte proliferation were assessed either by counting cells after trypsinization using a hemocytometer or by [^{3}H]TdR uptake as described previously (Vardy et al., 1995).

Pulse labeling and immunoprecipitation

Keratinocyte monolayers were washed once in cysteine/methionine-deficient DMEM medium and pre-incubated for 1 hour in this medium supplemented with 2% dialyzed fetal calf serum and anti-EGF-R antibody (15-6A or 425 at 10 μg/ml). Cultures were then incubated for an additional 3 hours in the same medium supplemented with 100 μCi/ml [^{35}S]methionine and [^{35}S]cysteine (Expre^{35}S^{35}S-labeling mix, Dupont-NEN; Boston, MA). Then cells were washed twice with ice-cold PBS without Ca^{2+} and Mg^{2+} and extracted for 20 minutes at 4°C with 3 ml per 10 cm dish of cold RIPA-buffer (0.5% desoxycholate, 0.1% SDS, 1.0% Triton-X-100, 150 mM NaCl, 50 mM Tris-Cl, pH 7.4, 0.5 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin). Nuclei and cellular debris were pelleted by centrifugation at 1,500 ***g*** for 2 minutes. Lysates were then centrifuged at 15,000 ***g*** for 30 minutes and frozen at −70°C. For preclearing, lysates were rotated with Protein A-Sepharose beads (Pharmacia, Piscataway, NJ) for 30 minutes at 4°C; Protein A beads were removed by spinning lysates at 16,000 ***g*** for 10 minutes. Aliquots of the precleared supernatants were counted in a beta counter. For immunoprecipitation, a volume corresponding to 1×10^7 cpm of precleared lysate was incubated with primary antibody: 1 μg of antibody (anti-α2, α5, α6-integrin-antibodies); or 1 μl of ascites fluid (anti-α1, α4-integrin-antibodies); or 30 μl of hybridoma supernatant (anti-α3), together with 1 μg secondary antibody (goat anti-mouse IgG; Pierce, Rockford, IL) and Protein A-Sepharose beads. After rotating for 2 hours at 4°C, Sepharose-bound immune complexes were harvested by centrifugation at 16,000 ***g*** for 2 minutes followed by 5 washes in 0.8 ml DOC-buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% desoxycholate). The pellets were boiled in Laemmli sample buffer and centrifuged; the supernatants were resolved by SDS-PAGE using a 4% stacking and 6% resolving gel. Protein gels were fixed for 30 minutes in 10% acetic acid and 25% isopropyl alcohol, dried and exposed to PhosphoImager™ screens (Molecular Dynamics, Sunnyvale, CA). Radioactive signals were analyzed using the PhosphoImager 445 SI Scanning system and Image QuaNT Software (Molecular Dynamics).

Flow cytometric determination of integrin expression

Flow cytometric analysis of cell surface integrin expression was performed as described previously (Rodeck et al., 1987). Briefly, keratinocyte cultures were detached using a trypsin/EDTA solution and trypsin was inactivated by washing cells in 15% FCS in PBS followed

by 2 washes in reaction buffer (PBS without Ca^{2+} and Mg^{2+} containing 1% BSA). Incubation with primary antibodies was for 45 minutes followed by 2 washes in reaction buffer and incubation with secondary antibodies, i.e. FITC-conjugated goat anti-mouse $F(ab')_2$ or goat anti-rat $F(ab')_2$ for 45 minutes. After 2 additional washes in reaction buffer, flow cytometric analysis was performed using an Ortho 2250 Cytofluorograph within 1 hour after sample preparation. All incubations and washes were performed at 4°C.

RESULTS

Induction of keratinocyte apoptosis by blockade of the EGF-R

To determine the effect of EGF-R blockade on human keratinocytes, early passage cultures were plated in serum-free medium in the presence of antagonistic anti-EGF-R mAb 425 or control mAb 15-6A at 10 μg/ml. When examined 1-5 days after seeding, these cultures revealed no effect of mAb 425 on keratinocyte viability, as determined by trypan blue exclusion, or on attachment and spreading, as determined by phase contrast microscopy. However, exposure to mAb 425 resulted in severe growth inhibition (50-90% after 5 days, depending on cell donor), as described previously (Vardy et al., 1995).

When keratinocyte cultures that had been exposed to mAb 425 for 3-5 days were passaged, they demonstrated a large reduction in their ability to reattach to fresh tissue culture-treated plastic dishes; specifically, we observed a 70-95% inhibition of attachment as compared to control cultures (Fig. 1). The small percentage of mAb 425-pretreated keratinocytes that did attach did not spread. Time course experiments demonstrated that continuous exposure to mAb 425 for at least 2 days was required to reduce reattachment and spreading of keratinocytes upon passaging. However, the reduction of attachment/spreading occurred regardless of whether mAb 425 was present in the medium during the 24 hour period after reseeding, indicating that the pretreatment period with mAb 425 had induced the subsequent inability to attach and spread. Lack of reattachment/spreading was observed after detachment of the mAb 425-pretreated keratinocytes with either a 2.5% trypsin/EDTA solution or a non-proteinaceous cell dissociation solution (Sigma; C5789), indicating that selective proteolysis of mAb 425-pretreated cultures was not a determining factor in the lack of adhesive ability. To determine if the mAb 425 effect on attachment was specific for epithelial cells, we tested several other normal human cell types that expressed EGF-R, including melanocytes and fibroblasts (MRC-5 and early passage dermal fibroblasts); there was no effect of mAb 425 on attachment and spreading of these other cell types (Fig. 1).

Under phase contrast microscopy, the unattached cells in

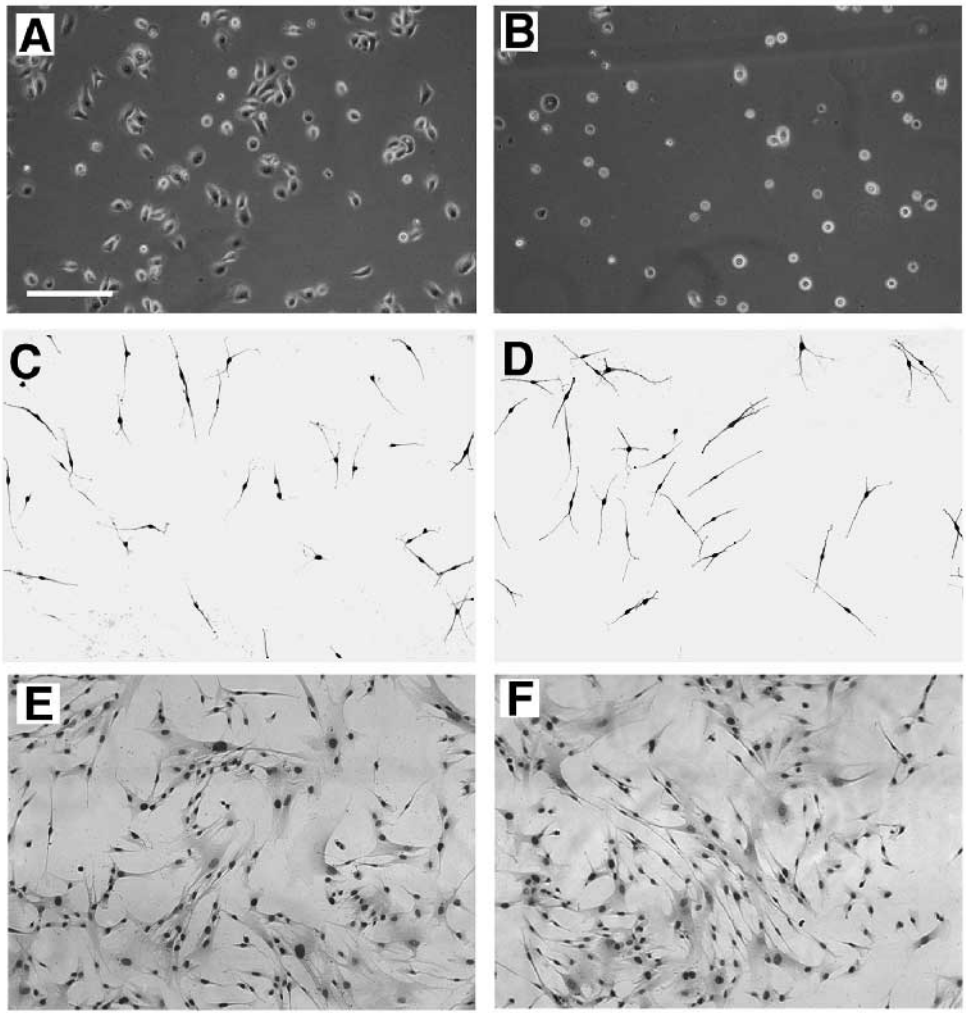

Fig. 1. Morphology of keratinocytes and other normal human cell types pretreated with EGF-R antagonistic mAb 425 and then passaged. Cells were exposed to either control mAb (A,C,E) or mAb 425 (B,D,F) for 4 days, detached from the culture dishes by trypsinization, and incubated for another 18-24 hours. (A and B) keratinocytes; (C and D) human neonatal melanocytes; (E and F) MRC-5 human lung fibroblasts. (A and B) Live cells as observed in phase contrast microscopy; all other panels show cells after fixation in 1% paraformaldehyde followed by staining with crystal violet. Note that mAb 425 pretreatment affects specifically keratinocyte attachment and morphology, having no obvious effect on melanocytes or fibroblasts. Bar, 100 μm.

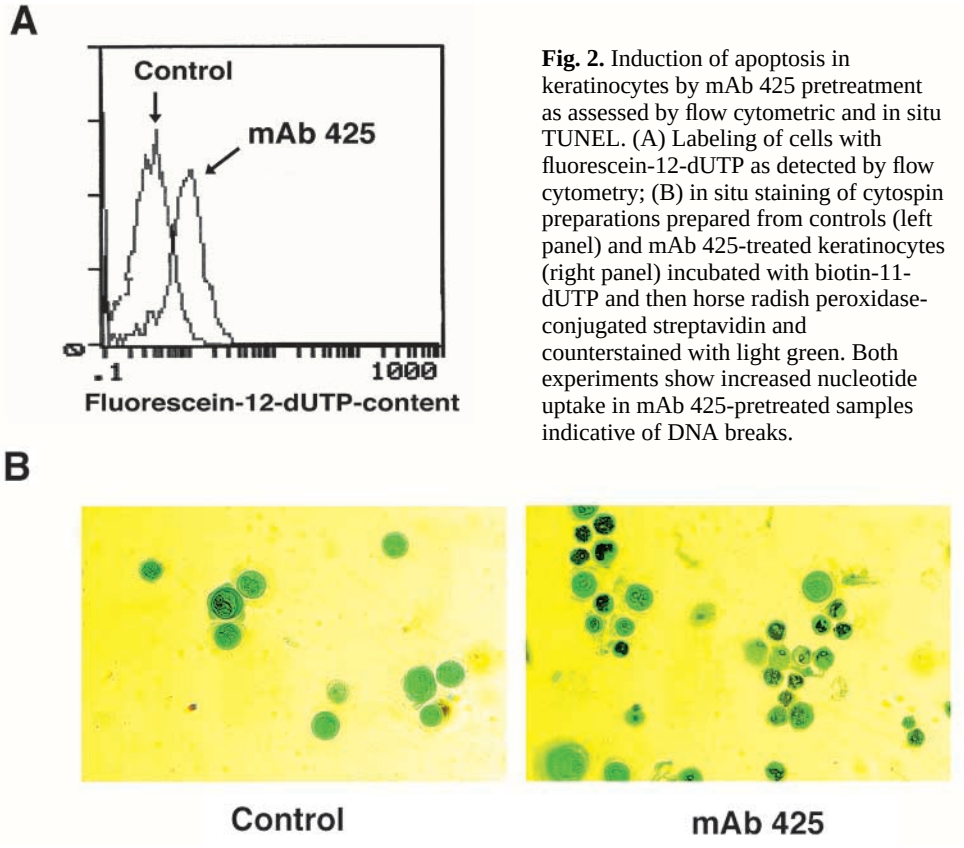

Fig. 2. Induction of apoptosis in keratinocytes by mAb 425 pretreatment as assessed by flow cytometric and in situ TUNEL. (A) Labeling of cells with fluorescein-12-dUTP as detected by flow cytometry; (B) in situ staining of cytospin preparations prepared from controls (left panel) and mAb 425-treated keratinocytes (right panel) incubated with biotin-11-dUTP and then horse radish peroxidase-conjugated streptavidin and counterstained with light green. Both experiments show increased nucleotide uptake in mAb 425-pretreated samples indicative of DNA breaks.

mAb 425-pretreated and passaged cultures appeared as floating cell bodies with intact, sharply demarcated cell borders, granular cytoplasm, and often condensed nuclei, a morphology consistent with apoptotic cell death. We therefore performed several assays to determine whether the mAb 425-pretreated, unattached cells had indeed undergone apoptosis, including: (i) TdT-mediated dUTP-biotin nick end-labeling (TUNEL) analysis, which is a measure of nicked DNA at the single cell level; (ii) flow cytometric analysis of propidium iodide-stained cells, which permits quantitative determination of the percentage of cells with subG_1 DNA content; (iii) acridine orange staining, which allows visualization of condensed and fragmented nuclei; and (iv) electron microscopy, which permits detection of condensed chromatin at the ultrastructural level. Flow cytometric and in situ TUNEL analyses showed that mAb 425-pretreated, unattached populations contained a high proportion of cells with damaged DNA as evidenced by the higher content of fluorescein-12-dUTP or biotin-11-dUTP in the mAb 425-pretreated cultures (Fig. 2). Quantification of the flow cytometric data, along with propidium iodide and acridine orange staining data for a single representative culture is given in Table 1. Although some variation in the percentage of apoptotic cells, depending on the assay method and the culture donor was observed, the mAb 425-pretreated cultures 24 hours after passaging consistently showed 7- to 13-fold more cells with altered DNA than the control cultures. The above evidence for DNA damage characteristic of apoptosis was confirmed by electron microscopy. As shown in Fig. 3, control keratinocytes exhibited a smooth nuclear membrane and an even, slightly granular nucleoplasm; in contrast, many of the cells in the mAb 425-pretreated and passaged cultures contained cells with condensed chromatin and fragmented nuclei considered typical of apoptotic cells. The mAb 425-pretreated and passaged cells were not proliferative, as evidenced by their lack of uptake of [^{3}H]TdR. Even after 10-14 days they did not regain viability or proliferative potential, remaining unattached and gradually disintegrating into cell debris.

The effect of mAb 425 on keratinocyte attachment and survival was likely due to inhibition of EGF-R activation, as indicated by the following control findings: (i) isotype-matched mAb 15-6A, which recognizes the carbohydrate

Table 1. Induction of apoptosis by mAb 425 treatment and passaging of keratinocytes

	Apoptotic cells (%)		
Condition	AO* staining	TUNEL staining†	PI staining‡
Control	6	9	4
mAb 425	43	69	55

*AO, acridine orange. Cells with irregularly shaped nuclei and/or condensed, brightly stained nuclei were scored positive.

†TUNEL staining of cytospin preparations examined by light microscopy (see Fig. 2).

‡Propidium iodide staining followed by flow cytometric analysis to quantify cells with sub-G1 DNA content.

Keratinocytes were incubated in the presence or absence of mAb 425 (10 μg/ml) for 5 days in defined MCDB medium free of EGF, followed by passaging and reseeding in the same medium but without mAb 425. Samples were prepared and analyzed 24 hours after passaging. Whereas the majority (>80%) of mAb 425-pretreated cells were not attached, almost all control cells (>90%) were attached. For all experimental conditions, floating and attached cells were combined. Results of a representative experiment are shown.

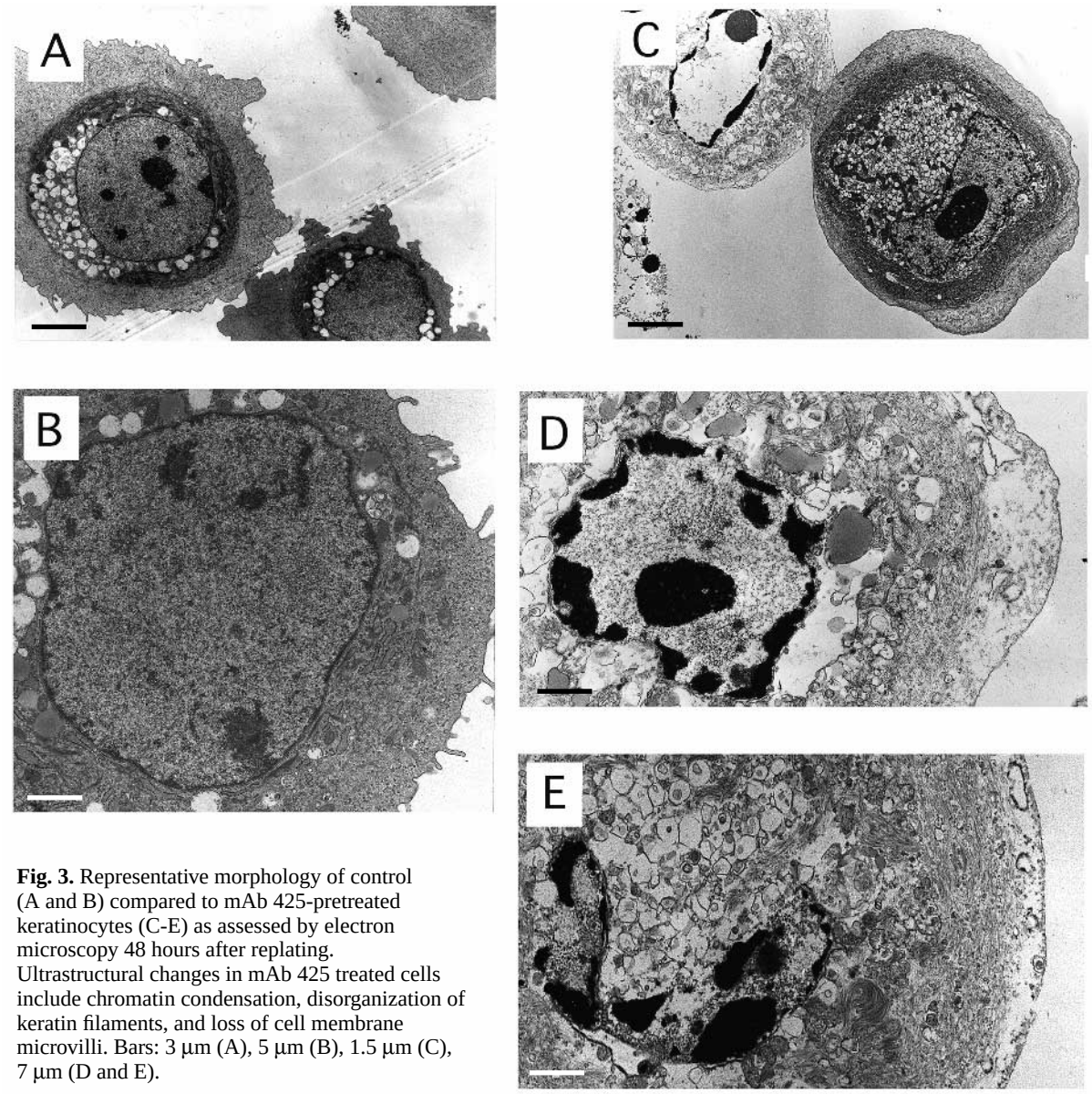

Fig. 3. Representative morphology of control (A and B) compared to mAb 425-pretreated keratinocytes (C-E) as assessed by electron microscopy 48 hours after replating. Ultrastructural changes in mAb 425 treated cells include chromatin condensation, disorganization of keratin filaments, and loss of cell membrane microvilli. Bars: 3 μm (A), 5 μm (B), 1.5 μm (C), 7 μm (D and E).

determinant Y expressed on the EGF-R and other proteins of epithelial cells (Basu et al., 1987) but does not affect EGF-R ligand binding or keratinocyte proliferation, did not affect keratinocyte adhesion or survival. (ii) An IGF-1 receptor antagonistic antibody (IR-3) that was used previously to inhibit insulin/IGF-1-dependent keratinocyte proliferation (Vardy et al., 1995) did not affect keratinocyte reattachment. (iii) As expected for an antagonist type inhibitor, the effect of mAb 425 pretreatment was overcome by high concentrations (50 ng/ml) of EGF. (iv) Pretreatment of keratinocytes for 3-5 days with TGF-β1 (2.5 ng/ml), which inhibited growth to approximately the same degree as mAb 425 at 10 μg/ml, did not inhibit attachment or viability of keratinocytes upon passaging, indicating that the induction of apoptosis by mAb 425 was not simply secondary to inhibition of cell growth. (v) Inhibition of the EGF-R tyrosine kinase activity using the tyrphostin AG1478 which has high affinity for the EGF-R (Levitzki and Gazit, 1995; Osherov and Levitzki, 1994) prevented keratinocyte adhesion upon passaging, with an ED_{50} of 10 nM (Fig. 4). At this concentration, AG1478 effectively inhibits phosphorylation of the EGF-R but not of the related c-erbB2 tyrosine kinase or the platelet-derived growth factor receptor (Levitzki and Gazit, 1995). The diluent DMSO had no effect on either cell growth or reattachment at the concentration range shown in Fig. 4.

To test whether adherent (i.e. not passaged) mAb 425-pretreated keratinocytes that show no signs of apoptosis were irreversibly committed to die, we investigated whether, in the absence of passaging, the effect of mAb 425 pretreatment was reversible. Keratinocytes were exposed to mAb 425 for 4 days and then incubated for an additional 3 days in medium free of mAb 425 and supplemented with EGF (10 ng/ml); trypsinization and reseeding at this time revealed only a slight reduction (18-25% in three separate experiments) in the ability of mAb 425-pretreated cells to reattach, demonstrating substantial reversibility of the mAb 425 effect. Consistent with their ability to reattach, these cells showed no morphological evidence of apoptosis. By comparison and as a control, replicate cultures

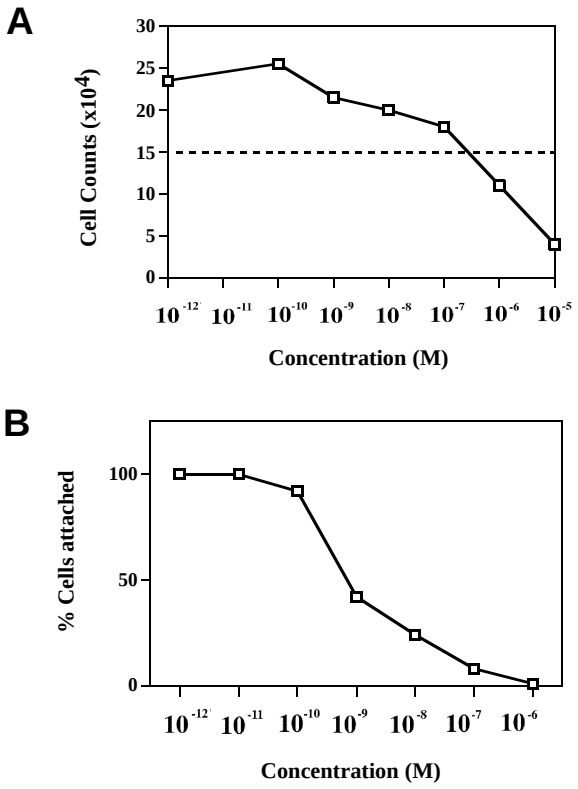

Fig. 4. Effects of tyrphostin AG1478 on keratinocyte growth and survival. (A) Cell counts of a keratinocyte culture maintained for 3 days in the presence of tyrphostin AG1478 at the concentrations indicated; results of one representative experiment of two are shown. Half maximal growth inhibition was achieved at approximately 10 nM AG1478. The broken line indicates the number of cells (1.5×10^4/well)seeded on day 1. (B) The effect of AG1478 on keratinocyte reattachment. Keratinocytes were preincubated with AG1478 for 3 days, followed by passaging and replating; the percentage of cells attached 24 hours after passage relative to control cells not preincubated with AG1478 is shown as a function of AG1478 concentration. Significant reduction in the ability to reattach was apparent at 1-10 nM AG1478. Results of one representative experiment of three are shown.

passaged after 4 days of continuous mAb 425-pretreatment showed an 85% reduction of reattachment and 65% of the unattached cells revealed nuclear changes consistent with apoptosis when examined by acridine orange staining 24 hours after splitting.

These results show that the effects of short-term treatment (i.e. 3-4 days) with mAb 425 on keratinocyte survival are largely reversible; however, long-term (up to 12 days) exposure to mAb 425 led to cumulative loss of cells due to rounding and shedding into the culture medium. The fraction of floating cells increased from <10% to 50% in mAb treated cultures between days 4 and 12. To exclude exhaustion of nutrients as a cause for this effect, the medium was replenished every 2nd day of the incubation period. Acridine orange staining of these cultures revealed nuclear condensation in many of the floating cells consistent with apoptosis. After 12 days, very few cells were still attached in the mAb-425-treated cultures, whereas control cultures were confluent. Upon reaching confluency, control cultures also exhibited an increase in the number of floating cells which, however, did not exceed 20% of the total population.

These data thus show that blockade of the EGF-R for several days, followed by detachment of the keratinocytes from the ECM leads to induction of cell death characterized by several features of apoptosis. Blockade of the EGF-R thus seems to prime the keratinocytes for induction of apoptosis by a second signal, i.e. detachment from the ECM. Based on these data, we hypothesize that both EGF-R activation and ECM attachment provide important signals for maintenance of keratinocyte survival; when signals from both of these receptors are interrupted, large scale apoptosis is induced.

Effects of EGF-R blockade on integrin expression and function

Previous experiments with other epithelial cells have shown that physical inhibition of attachment to the substratum is sufficient to induce apoptosis (Frisch and Francis, 1994). In light of these findings, we considered the hypothesis that blockade of the EGF-R might lead to lack of attachment through effects on the expression or function of integrins, thereby inducing apoptosis. We therefore determined the effect of mAb 425 on integrin biosynthesis, steady-state level, and function in keratinocyte cultures. To quantify integrin biosynthesis, control and mAb 425 treated keratinocytes were pulse labeled with radioactive amino acids, extracted, and subjected to immunoprecipitation with specific antibodies against integrin subunits. After 3 days of mAb 425 treatment, only a small inhibitory effect on synthesis of the α2, α3, α5, and α6 integrins was noted (Fig. 5). In agreement with earlier studies (Adams and Watt, 1991), the α1 and α4 integrin subunits were not expressed in our keratinocyte cultures. Flow cytometric analysis confirmed that keratinocytes treated with mAb 425 for 3 days expressed similar levels of cell surface integrins as control cells (Table 2).

To address whether integrin function in mAb 425-treated keratinocytes was impaired, we measured de novo attachment of mAb 425-pretreated cells onto endogenous keratinocyte-derived ECM, which is primarily laminin 5 (epiligrin). Laminin 5, a component of the basement membrane, is a ligand for integrins α3β1 and α6β4, both of which are expressed by normal keratinocytes (Carter et al., 1991). In contrast to tissue culture-treated plastic (see Fig. 1), laminin 5 supported almost

Table 2. Cell surface integrin expression by keratinocytes in the presence of mAb 425

	Control		mAb 425	
Integrin antibody	% Positive cells	MFI*	% Positive cells	MFI
None	4.6	68.6	5.8	70.3
α2	92.5	91.7	78.4	73.4
α3	98.4	97.7	92.6	85.4
α5	80.8	59.3	64.4	68.2
α6	55.7	90.4	42.4	94.6
β1	98.1	93.2	92.5	91.3

Keratinocytes were incubated in the presence or absence (control) of mAb 425 for 3 days. They were then removed from the culture dish with trypsin, incubated with antibody to the indicated integrin subunits, and subjected to flow cytometry to quantify the percentage of cells expressing integrin subunits on their surface and their mean fluorescence intensity (MFI). Results of one representative experiment of three are shown.

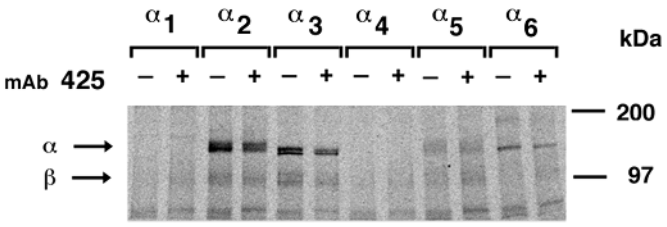

Fig. 5. Integrin biosynthesis in keratinocytes. Cells were treated with mAb 425 or with control mAb 15-6A for 3 days, followed by pulse-labeling with [^{35}S]methionine and [^{35}S]cysteine for 3 hours. Integrin biosynthesis was assessed by immunoprecipitation with indicated antibodies to integrin α subunits and SDS-polyacrylamide gel electrophoresis and autoradiography of the immunoprecipitated material. The position of α and coprecipitated β integrin subunits is marked by arrows on the left and the position of molecular mass markers is on the right. Keratinocytes in culture synthesize α2, α3, α5, and α6 but not α1 or α4. Addition of mAb 425 has only a small inhibitory effect on integrin biosynthesis.

complete reattachment and spreading of mAb 425-pretreated keratinocytes within 1 hour after seeding, similar to control cells (Fig. 6). However, while control cells remained attached, many of the mAb 425-pretreated cells became rounded and then detached within 24 hours after reseeding. Table 3 summarizes data from 3 experiments in which keratinocytes were plated onto plastic or matrix-coated plates, incubated further for 24-48 hours, and then trypsinized and counted. Plating on laminin 5 increased the number and percentage of cells recovered from both control and mAb 425-pretreated cultures; however, the percentage increase in recovery was greater for mAb 425-pretreated cells. These results indicate that plating on endogenous matrix effected a partial, but far from complete, recovery from the mAb 425 effect, even though initially nearly all cells adhered and spread.

Table 3. Reattachment of mAb 425-pretreated keratinocytes upon replating on keratinocyte-derived extracellular matrix

Pretreatment	Replated on	No. of cells ($\times 10^5$) attached	% Recovery
Experiment 1			
Control	Plastic	0.95	63
mAb 425	Plastic	0.32	21
Control	ECM	1.40	93
mAb 425	ECM	0.68	45
Experiment 2			
Control	Plastic	0.88	59
mAb 425	Plastic	0.25	17
Control	ECM	0.97	67
mAb 425	ECM	0.45	30
Experiment 3			
Control	Plastic	2.4	240
mAb 425	Plastic	<0.1	<10
Control	ECM	3.2	320
mAb 425	ECM	0.8	80

Keratinocytes were incubated in the presence of mAb 425 for 4 days. They were then trypsinized, counted, and replated on tissue culture-treated plastic or on plates coated with endogenous keratinocyte matrix (ECM). After a further incubation of 24 hours (exp. 1 and 2) or 48 hours (exp. 3) cells were counted and % recovery calculated based on the number of cells seeded.

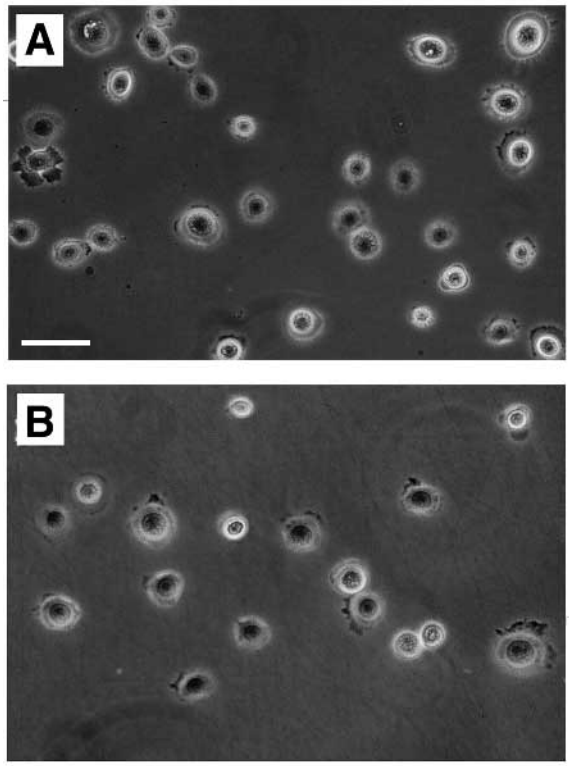

Fig. 6. Short-term attachment and spreading of keratinocytes on laminin 5 is not affected by mAb 425 pretreatment. Control (A) and mAb 425-pretreated (B) cells were seeded on keratinocyte-derived ECM and incubated for 6 hours. Both populations are attached and spread with prominent lamellopodia. Bar, 30 μm.

To assess strictly and quantitatively the viability of recovered cells, the cultures were pulsed with [^{3}H]TdR. As shown in Table 4, the mAb 425-pretreated cells that remained adherent to endogenous matrix synthesized DNA at the same rate as controls, when measured on a per cell basis. By contrast and as expected, mAb 425-pretreated cells seeded on plastic did not incorporate [^{3}H]TdR.

Taken together, these results indicate that short-term integrin expression and function are not impaired by mAb 425 treatment; furthermore, integrin engagement by matrix can partially rescue keratinocyte apoptosis induced by EGF-R blockade. However, long-term keratinocyte adhesion is compromised by mAb 425-treatment in a substantial proportion of the cells.

Table 4. Rescue of mAb 425-pretreated keratinocytes upon reseeding on keratinocyte-derived extracellular matrix

Pretreatment	Re-plated on	^{3}H-TdR total	^{3}H-TdR/ 1×10^5 cells
Control	ECM	3135	1000
mAb 425	ECM	790	990
mAb 425	Plastic	96	<96

Keratinocytes were grown in the presence and absence of mAb 425 for 4 days. They were then passaged and replated on keratinocyte-derived ECM or plastic tissue culture plates. After 48 hours of further incubation, an [^{3}H]TdR pulse was given for 8 hours prior to harvest.

DISCUSSION

This study provides evidence that blockade of the EGF-R by mAb 425 significantly shortens the life span of cultured normal human keratinocytes by inducing a cell death program displaying several features of apoptosis. Many previous studies have shown that activation of the EGF-R mediates proliferation and migration of epithelial cells (Ando and Jensen, 1993; Chen et al., 1993, 1994; Coffey et al., 1987; Cook et al., 1991b, 1992; Pittelkow et al., 1993; Vardy et al., 1995). However, our studies are the first to show that activation of the EGF-R also serves another basic function in normal epithelial cells, i.e., protection from apoptosis. The necessity of EGF-R activation for survival appears to be cell-type specific, since neither adhesion nor survival of normal melanocyte or fibroblast cultures, both of which express EGF-R, is affected by treatment with the EGF-R antagonistic antibody mAb 425.

Several previous studies have demonstrated that normal epithelial cells must adhere to a substratum in order to survive. For example, apoptosis is induced in an immortalized human keratinocyte cell line when it is prevented from attaching to substratum (Frisch and Francis, 1994). In addition, apoptosis of mammary epithelial cells is accelerated by inhibition of β1 integrin-mediated binding to basement membrane (Boudreau et al., 1996; Pullan et al., 1996), and integrins have recently been implicated in protection of human endothelial cells from apoptosis (Meredith et al., 1993). We therefore investigated whether blockade of the EGF-R may inhibit adhesion by modulating integrin expression or function, thereby inducing apoptosis. Our data do not support this mechanism. Specifically, after 3 days of mAb 425 exposure, there are only slight decreases in integrin synthesis and cell surface expression. Furthermore, mAb 425-pretreated cells attach and spread normally on endogenous keratinocyte matrix, when examined in short-term (30 minutes to 4 hours) assays. These results are consistent with the view that mAb 425 treatment does not induce apoptosis primarily by inhibiting integrin function and thus blocking attachment. Rather we suggest that blockade of the EGF-R primes the keratinocyte for susceptibility to a second signal, which is generated via detachment from ECM during the splitting procedure and is rapidly followed by apoptosis.

Our findings strongly imply cooperativity between EGF-R activation and integrin engagement with respect to the maintenance of normal epithelial cell survival. In support of overlapping functions mediated by EGF-R and integrins, we have observed reversal of apoptosis in a subpopulation of mAb 425-pretreated keratinocyte cultures when such cells are seeded on endogenous substrate, which affords rapid integrin-mediated reattachment after splitting. The rescued cell population is viable as demonstrated by the fact that they incorporate [^{3}H]TdR to the same extent as control cultures. However, it must be noted that full reversal of the apoptotic phenotype is not achieved, indicating that at least under our experimental conditions, the contributions of the EGF-R and of integrins to survival are not totally redundant. Our results extend an hypothesis recently advanced by Assoian and colleagues for coordinate regulation of the cell cycle by integrins and mitogens (Guadagno and Assoian, 1991; Zhu et al., 1996). These authors found that dual, non-redundant signaling through growth factor receptors and through integrins is required to sustain proliferation of normal anchorage-dependent fibroblasts. The integrin and growth factor receptor-dependent signaling pathways converge to modulate expression and/or function of G_1 cyclins and associated molecules (Zhu et al., 1996). We think it is likely that in the normal keratinocyte a similar situation exists, in that integrin and EGF-R pathways may interdigitate to control common down-stream molecular targets relevant to survival. For example, EGF-R blockade may interfere with one or more 'post occupancy' events of integrin activation, e.g. the phosphorylation of components of the focal adhesions which are required for long-term integrin function. Consistent with this idea, receptor tyrosine kinases have been shown to phosphorylate focal adhesion kinase (Chen and Guan, 1994) and talin (Tidball and Spencer, 1993) which participate in the proper assembly of focal adhesions and signaling upon integrin activation (Schlaepfer et al., 1994). It remains to be determined whether the EGF-R can catalyze phosphorylation of focal adhesion kinase or other focal adhesion components.

A recent report by Hines and Allen-Hoffmann (1996) has provided evidence that keratinocyte growth factor (KGF), a member of the fibroblast growth factor (FGF) family, may also act as a survival factor for keratinocytes. Specifically, these investigators showed that KGF-treated keratinocytes were less susceptible to apoptosis upon suspension in semi-solid medium. Based on these data, we performed experiments to determine if KGF (at 1-50 ng/ml) is able to overcome the effect of EGF-R blockade. Our findings demonstrated that addition of KGF does not affect survival of keratinocytes treated with mAb 425 (U. Rodeck, unpublished observations). Hence the mechanisms by which the EGF-R and the KGF-R control keratinocyte survival are not redundant.

In summary, our results establish that in normal keratinocytes EGF-R ligands serve a dual role in supporting both cell cycle progression and survival. An important topic for future investigations is the relevance of the EGF-R survival function to tumor development and spread. We think it is important to consider the possibility that upregulation of EGF-R autocrine loops in neoplastic epithelial cells not only may confer the ability to proliferate independently of exogenous growth factors but also may promote inappropriate survival by protecting them from apoptosis.

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