Epidermal growth factor receptor induced apoptosis: potentiation by inhibition of Ras signaling

Thorbergur Högnason, Sukalyan Chatterjee, Timothy Vartanian, Rajiv R. Ratan, Krista M. Ernewein, Amyn A. Habib*

Department of Neurology, Beth Israel Deaconess Medical Center and Harvard Medical School Boston, Boston, MA 02115, USA

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Abstract Previous studies have shown that certain tumor cell lines which naturally express high levels of the epidermal growth factor receptor (EGFR) undergo apoptosis when exposed to epidermal growth factor. Whether this phenomenon is a direct result of receptor overexpression or some other genetic alteration renders these cells sensitive to apoptosis is yet to be established. We show that experimentally increasing the level of EGFR expression predictably leads to apoptosis in a variety of cell types which requires an active tyrosine kinase but not EGFR autophosphorylation sites. Expression of a dominant negative Ras mutant in EGFR overexpressing cells results in a significant potentiation of EGFR induced apoptosis suggesting that Ras activation is a key survival signal generated by the EGFR. We propose that potentiation of EGFR induced apoptosis by dominant negative Ras results, at least in part, by a block of Akt activation. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Epidermal growth factor receptor; Apoptosis; Ras; Akt; Signaling; Growth factor

1. Introduction

The epidermal growth factor receptor (EGFR) is commonly overexpressed in cancer [1] and increased EGFR expression correlates with malignant progression. Paradoxically, however, ligand stimulation of cells which overexpress the EGFR has previously been shown to result in apoptosis [2,3]. Previous studies, however, have not addressed whether the high levels of receptor expression per se lead to ligand induced apoptosis or whether some other genetic alteration has rendered these cells abnormally sensitive to the effects of epidermal growth factor (EGF). It is also unknown if EGFR induced apoptosis is an unusual feature of certain tumor cell lines or whether it is a general consequence of EGFR overexpression.

Activation of the EGFR tyrosine kinase results in the generation of a number of intracellular signals which culminate in cell proliferation [4,5]. One key signal generated by the activated EGFR is the activation of p21 Ras [6], which in turn controls the activity of a number of downstream effectors exerting complex effects ranging from proliferation to differentiation and cell death [7]. Earlier studies had identified a key role for Ras in mitogenic signaling [8]. However, more recent studies suggest a more complex role of p21 Ras. For example, introduction of p21 Ras into primary cells results in growth arrest [9]. Ras signaling also plays a regulatory role in apoptosis, which may be pro- or antiapoptotic [7].

A kinase inactive EGFR mutant is unable to signal mitogenesis, but retains the ability to generate certain EGF induced signals such as mitogen activated protein kinase activation [10]. Interestingly, a mutant EGFR which lacks all five autophosphorylation sites retains the ability to signal mitogenesis [11]. This mutant receptor can activate p21 Ras and tyrosine phosphorylate Shc in response to EGF [12]. In this study we demonstrate that apoptosis is a direct and predictable outcome of EGFR overexpression in a variety of cell lines and explore the role of Ras signaling in EGFR induced apoptosis.

2. Materials and methods

2.1. Materials

2.1.1. Cell lines. 293 EBNA cells were obtained from Invitrogen (Carlsbad, CA, USA). R1hER cells were obtained from Dr. Michael Weber (Charlottesville, VA, USA). U-87 MG and C6 glioma cells were obtained from ATCC.

2.1.2. DNA. A human EGFR construct cloned into pCDNA 3.1 [13] was used for transient transfection experiments. A kinase inactive mutant EGFR, CMV Neo-HER M^{721}, was obtained from Dr. Axel Ullrich (Martinsried, Germany). A truncated EGFR mutant lacking all five major autophosphorylation sites was generated by PCR amplification of a 300 bp internal fragment of the EGFR gene introducing a stop codon before the first tyrosine residue (Y992). This fragment was cloned in pCDNA 3.1+ with the region of the EGFR gene upstream of the amplified sequence. A pSV-β-galactosidase control vector was obtained from Promega (Madison, WI, USA). pMMTV-rasH(Asn-17) and pMMTV v-Ras expression plasmids were obtained from Dr. Geoffrey Cooper (Boston, MA, USA), described in [14].

2.1.3. Antibodies. EGFR and phospho-Akt antibodies (05-104 and 06-801) were purchased from Upstate Biotechnology (Lake Placid, NY, USA). A pan Ras antibody (Clone Ras 10, OP40) was purchased from Oncogene Research Products (Cambridge, MA, USA). Anti-phospho ERK antibodies (Cat. No. V6671) were obtained from Promega (Madison, WI, USA). ERK2 and Akt antibodies (sc-154 and sc-8312) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Transfection

A standard calcium phosphate protocol was used for all transfections described in this study [15]. For creation of stable cell lines, R1hER cells were transfected using the calcium phosphate method and mass populations of G418 resistant cells were pooled and tested

*Corresponding author. Harvard Institutes of Medicine, Rm. 836, 77 Avenue Louis Pasteur, Boston, MA 02115, USA.
Fax: (1)-617-667 0811. E-mail: ahabib@caregroup.harvard.edu

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for expression of Ras by Western blotting following exposure to dexamethasone.

2.3. Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting were performed as previously described [13].

2.4. Assays for cell death

2.4.1. Morphological analysis. Cells were transfected with an expression vector for β-galactosidase (β-Gal) along with the EGFR or control vector, or with empty vector or EGFR without β-Gal, for the time periods indicated in the figure legends followed by X-Gal staining for cells transfected with β-Gal according to standard protocols [15]. Blue cells were defined as dead or alive based on the morphological criteria described in Section 3.

2.4.2. DNA laddering. DNA was extracted from cells using an Easy-DNA kit from Invitrogen (Carlsbad, CA, USA) according to the manufacturer's protocol and analyzed on a 2% agarose gel stained with ethidium bromide.

2.4.3. Cell death detection ELISA. A cell death detection ELISA kit (Cat. No. 1774425) was obtained from Boehringer Mannheim USA and used according to the manufacturer's protocol. This is a photometric enzyme immunoassay for the quantitative detection of cytoplasmic histone associated DNA fragments after induced cell death.

3. Results

3.1. Apoptosis is a predictable outcome of EGFR overexpression in various cell lines

R1hER cells are Rat-1 fibroblasts transfected with the human EGFR expressing about 7.5×10^5 EGF receptors, which is approximately a sevenfold increase over the parental Rat-1 cells [16]. When exposed to EGF, R1hER cells undergo apoptosis. In contrast, addition of EGF to Rat-1 cells results in a modest mitogenic effect without evidence of apoptosis. Addition of EGF to R1hER cells results in apoptosis detectable within 24 h with most cells dying within 48 h. While unstimulated R1hER cells grow to confluence, cells exposed to EGF show morphologic features of apoptosis including cell shrinkage, membrane blebbing, and nuclear condensation (Fig. 1A,B). DNA fragmentation, a cardinal feature of apoptosis, was evident in EGF treated R1hER cells but not in untransfected Rat-1 fibroblasts treated with EGF (Fig. 1D), further demonstrating that the EGF mediated cell death is apoptotic. In Fig. 1 R1hER cells were treated with EGF at a concentration of 100 ng/ml. Apoptosis can also be detected when these cells are treated with lower concentrations of EGF (Fig. 5A).

The transient overexpression of the EGFR also results in apoptosis in various cell lines. Using a β-Gal cotransfection assay which has been widely used to assess apoptosis in transiently transfected cells [17], we transiently overexpressed the EGFR along with a β-Gal gene in 293 EBNA cells. This resulted in the death of more than 90% of transfected 293 EBNA cells within 24 h (Fig. 2B), with cells displaying morphological alterations of adherent cells undergoing apoptosis, becoming rounded and condensed, and detaching from the dish. Cell death was not observed when the vector alone was cotransfected with β-Gal (Fig. 2A). Fig. 2C,D shows 293 cells transfected with vector alone or EGFR alone. Since

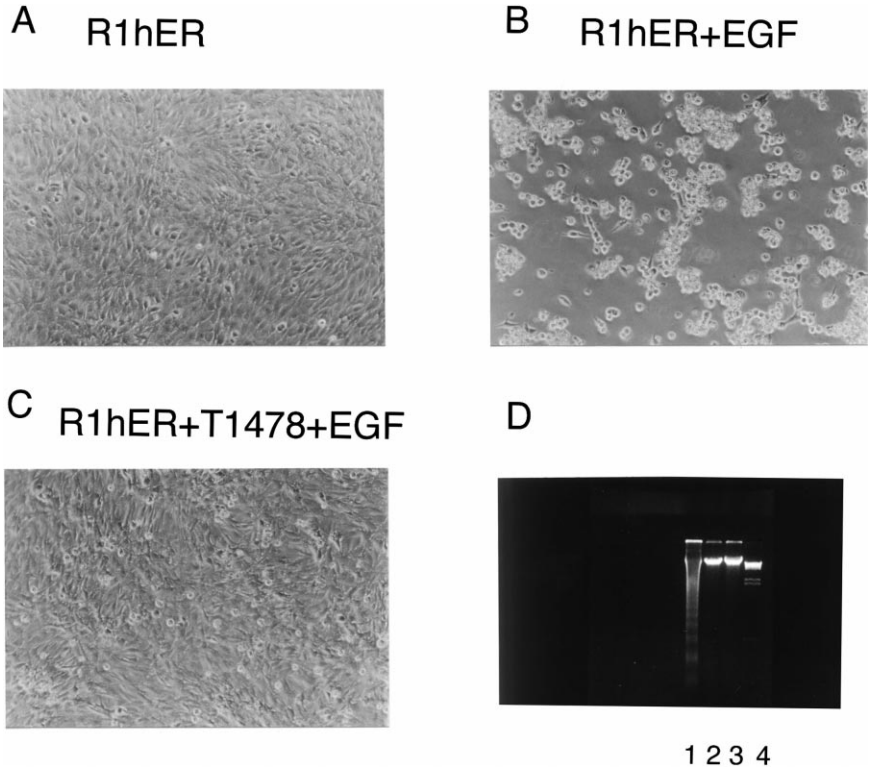

Fig. 1. EGF induces apoptosis in R1hER cells. EGF was added to cells in the presence of 10% fetal bovine serum for 48 h at a concentration of 100 ng/ml and cells were photographed under phase contrast microscopy. A: Unstimulated R1hER (Rat-1 cells overexpressing the EGFR) cells. B: R1hER cells stimulated with EGF. C: R1hER cells preincubated with tyrphostin AG 1478 (100 nM) for 2 h before addition of EGF for 48 h. No apoptosis could be detected in the parental Rat-1 cells in response to EGF (not shown). D: DNA fragmentation that results from EGF stimulation in R1hER cells but not in untransfected Rat-1 fibroblasts. Lane 1 shows R1hER cells treated with EGF, with the appearance of DNA laddering seen in apoptosis. Lane 2 shows unstimulated R1hER cells. Lane 3 shows Rat-1 fibroblasts treated with EGF. Lane 4 shows Lambda/*Hin*dIII molecular weight markers.

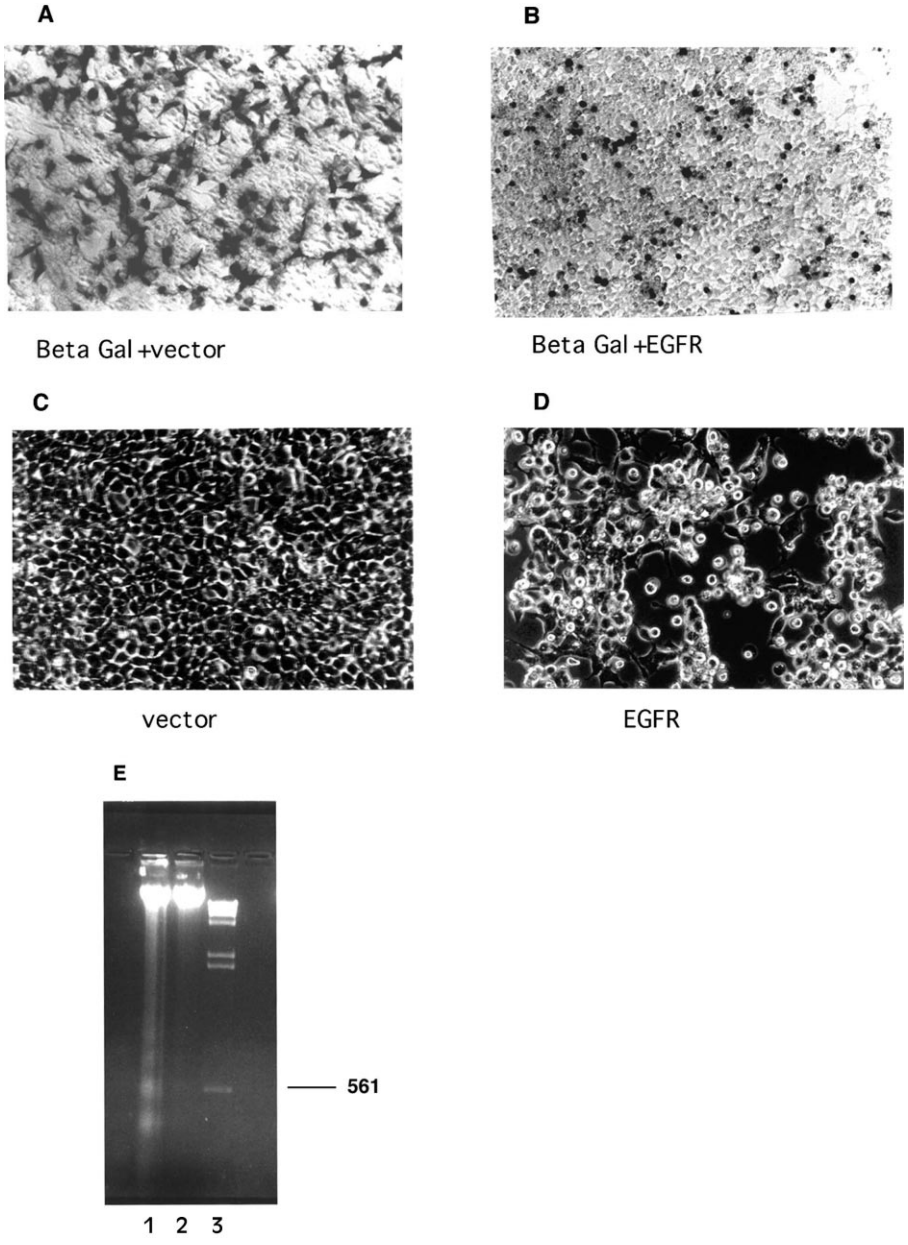

Fig. 2. EGFR overexpression induces a ligand independent apoptosis in 293 EBNA cells. Cells were plated overnight in Dulbecco's modified Eagle's medium/10% fetal bovine serum in 6 well dishes. Transfections were performed the next day using the calcium phosphate method. A: Cells were transfected with β-Gal (1 μg) plus an empty vector (4 μg) or EGFR. B: Cells were stained with X-Gal 24 h after transfection. C,D: Cells were transfected with an empty vector or EGFR (20 μg) and photographed after 48 h. E: DNA fragmentation in 293 cells transfected with the EGFR (lane 1), but not with the empty vector (lane 2). Lane 3 shows molecular weight markers.

our transfection efficiency in this cell line is high (about 30%), apoptosis can be detected morphologically in these cells even in the absence of β-Gal as a marker (Fig. 2D). Trypan blue staining of cells transfected with the EGFR showed that about 30% of cells failed to exclude the dye whereas 95% of cells transfected with the vector alone were viable (not shown). Expression of the EGFR was confirmed by immunoblotting in parallel experiments (not shown). We also observe DNA fragmentation in cells transfected with the EGFR but not in vector transfected cells, confirming that the cell death is apoptotic (Fig. 2E).

EGFR induced apoptosis in 293 EBNA cells is ligand independent due to maximal tyrosine phosphorylation of the EGFR upon ectopic expression in these cells. Addition of EGF does not result in a further increase in tyrosine phosphorylation of the receptor in these cells [18]. Ligand independent signaling by ectopically expressed EGFR has also been noted in 293T cells [19]. Overexpression of the EGFR also leads to ligand dependent apoptosis in U-87 MG cells and in C6 glioma cells (not shown).

Thus EGFR overexpression can lead to ligand dependent apoptosis in cell types as diverse as epithelial (MDA-MB-468, A431, and 293), mesenchymal (Rat-1 fibroblasts), and glial (U-87 MG and C6) cells.

3.2. *EGFR mediated apoptosis requires an active tyrosine kinase but not EGFR autophosphorylation sites*

To understand the molecular mechanism by which the over-

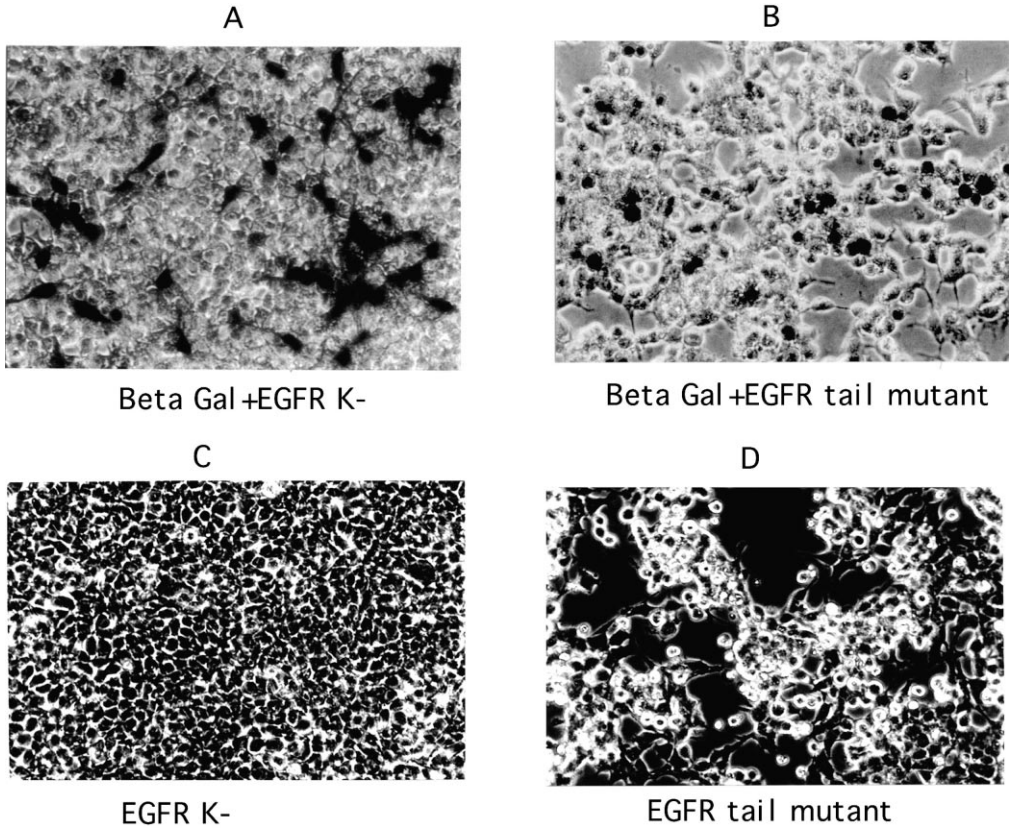

Fig. 3. EGFR mediated apoptosis requires an active tyrosine kinase but not EGFR autophosphorylation sites. A: Cells were transfected with β-Gal (1 μg) plus a kinase inactive EGFR mutant (4 μg). B: Cells were transfected with β-Gal plus a truncated EGFR mutant (4 μg) which lacks all five major autophosphorylation sites. Cells were stained with X-Gal 24 h after transfection. C,D: Cells were transfected with vector alone or EGFR respectively (20 μg) and photographed after 48 h.

expressed EGFR induces apoptosis we examined the ability of EGFR mutants to induce apoptosis. A mutant EGFR lacking intrinsic tyrosine kinase activity does not induce apoptosis in transiently transfected 293 cells (Fig. 3A,C). Pretreatment of R1hER cells with tyrphostin AG 1478, a specific EGFR kinase inhibitor, also blocks EGF mediated apoptosis in R1hER cells (Fig. 1C). However, the EGFR with intact tyrosine kinase activity but lacking its five autophosphorylation sites, hence lacking the putative binding sites for SH2 domain proteins, potently induces apoptosis in these cells (Fig. 3B,D).

3.3. A dominant negative Ras mutant potentiates apoptosis induced by the EGFR: a possible role of Akt

Since the truncated EGFR lacking all five major autophosphorylation sites is competent to generate the apoptotic signal and previous studies have shown that this mutant receptor retains the ability to activate Ras in response to mitogenic signals, we tested whether a key apoptotic signal was transmitted through p21 Ras by creating created stable R1hER cell lines expressing a dominant negative Ras (N17) mutant under the control of a dexamethasone inducible promoter. To confirm that the transfected dominant negative Ras construct was functional we verified that EGF induced ERK activation is significantly attenuated in cells transfected with dominant negative Ras (Fig. 4B) compared to empty vector. We find that EGF induced apoptosis is dramatically increased in cells transfected with a dominant negative Ras (Fig. 5B). The results of a quantitative photometric ELISA for DNA fragmentation (Fig. 5C) also demonstrate an increase in EGF mediated apoptosis in cells expressing dominant negative Ras.

Previous studies have shown that activation of phosphatidylinositol 3-kinase (PI 3-kinase) is Ras dependent [20]. Activation of PI 3-kinase results in activation of protein kinase B/Akt which is known to be a key antiapoptotic signal [21]. We find that Akt activation is blocked in R1hER cells expressing a dominant negative Ras mutant but not in R1hER cells expressing the empty vector (Fig. 5D). This suggests that the effect of dominant negative Ras in potentiating EGFR mediated apoptosis is at least in part due to a block of Akt activation. The EGFR is functional in R1hER cells expressing dominant negative Ras since EGF induced activation of STAT1 is not attenuated in these cells compared to R1hER cells expressing empty vector (not shown).

4. Discussion

While previous studies have shown that EGF induces apoptosis in certain tumor cells lines which naturally overexpress the EGFR it has not been rigorously established that this apoptosis is a direct result of EGFR overexpression. In this study we show that transient or stable overexpression of the EGFR predictably leads to ligand dependent apoptosis in a variety of cell lines suggesting that apoptosis may be a general outcome of EGFR overexpression. A previous study has also reported that stimulation of tumor cells overexpressing the

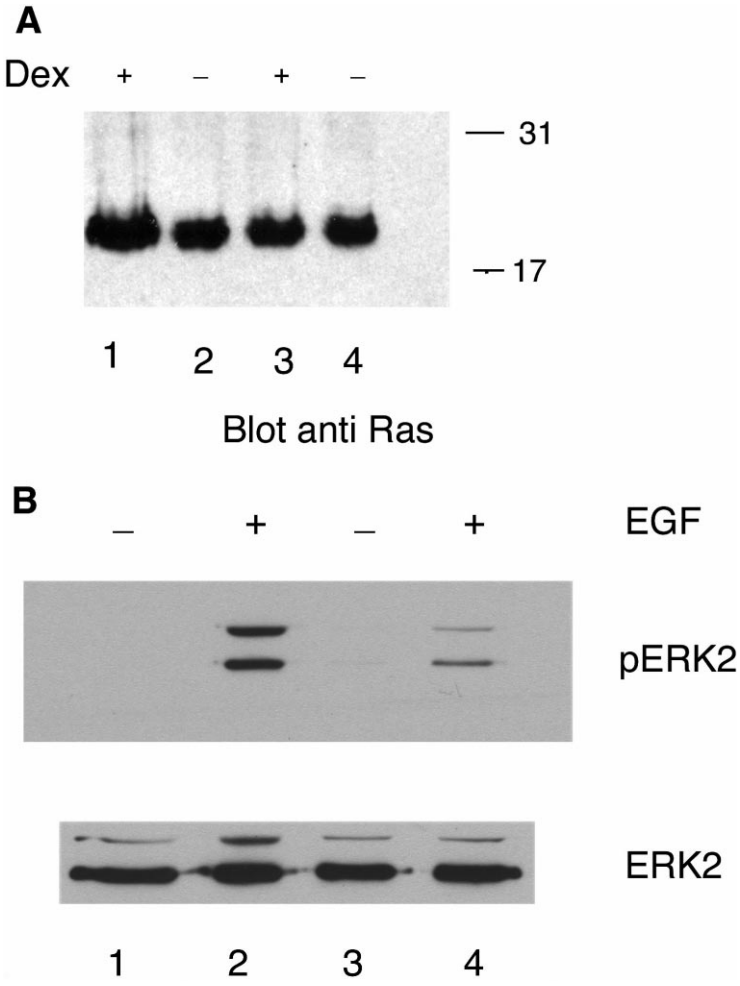

Fig. 4. Expression of a dominant negative Ras mutant potentiates EGF induced apoptosis. A: Cells transfected with dominant negative Ras (lanes 1 and 2) in which an increase in Ras levels can be detected upon exposure to dexamethasone (1 μM) for 16 h while such an increase is not detected in the vector transfected R1hER cells (lanes 3 and 4). B: Cells expressing dominant negative Ras were tested for their ability to activate ERK2 (using a phosphospecific antibody) in response to EGF following exposure to dexamethasone. Cells expressing dominant negative Ras show a smaller increase in ERK activation in response to growth factor (lanes 3 and 4) compared to vector transfected cells (lanes 1 and 2). The blot was stripped and reprobed with ERK2 antibodies to determine protein loading (lower panel).

ErbB2 receptor with Heregulin induces apoptosis [22] suggesting that apoptosis may be an outcome of high levels of expression of other receptor tyrosine kinases as well.

The exact level of EGFR expression where a mitogenic stimulus changes into a death stimulus probably varies depending on the cell type. A431 cells and MDA-MB-468 cells express about 2×10^6 receptors [23] while R1hER cells express about 7.5×10^5 receptors, about a sevenfold increase over untransfected Rat-1 fibroblasts.

EGFR mediated apoptosis, like mitogenic signaling, requires an active kinase but not EGFR autophosphorylation sites, indicating that the truncated receptor can generate the apoptotic signal. Since in previous studies the truncated receptor has been shown to activate p21 Ras, a critical component of mitogenic signaling, we studied the effect of inhibiting Ras signaling on EGF mediated apoptosis. We find that inhibiting Ras signaling results in a significant potentiation of EGFR induced apoptosis.

p21 Ras has recently been discovered to be a key modulator of apoptosis induced by other stimuli as well. For example, Ras activation may be required for apoptosis induced by the tumor necrosis factor and by Fas [24,25]. Activated Ras may render cells sensitive to apoptosis induced by downregulation of protein kinase C [26]. Activation of p21 Ras also results in the activation of the antiapoptotic PI 3-kinase/Akt pathway. We find that Akt activation is significantly impaired in cells expressing dominant negative Ras suggesting that the potentiation of EGFR induced apoptosis in these cells may result from impaired Akt activation. Attenuation of ERK activity may also contribute to the increased apoptosis seen in cells expressing dominant negative Ras, since ERK activity has previously been shown to be antiapoptotic [27].

While the mechanism of EGFR induced apoptosis remains unknown a recent study has proposed that EGFR mediated apoptosis requires detachment of cells from the substratum [28]. This was based in part on finding evidence of caspase activation only in detached MDA-MB-468 cells following EGF treatment. Our study does not support the hypothesis that cell detachment is required for EGFR mediated apoptosis. All our experiments were performed in adherent cells and we consistently found both morphological and biochemical evidence of apoptosis in adherent cells treated with EGF.

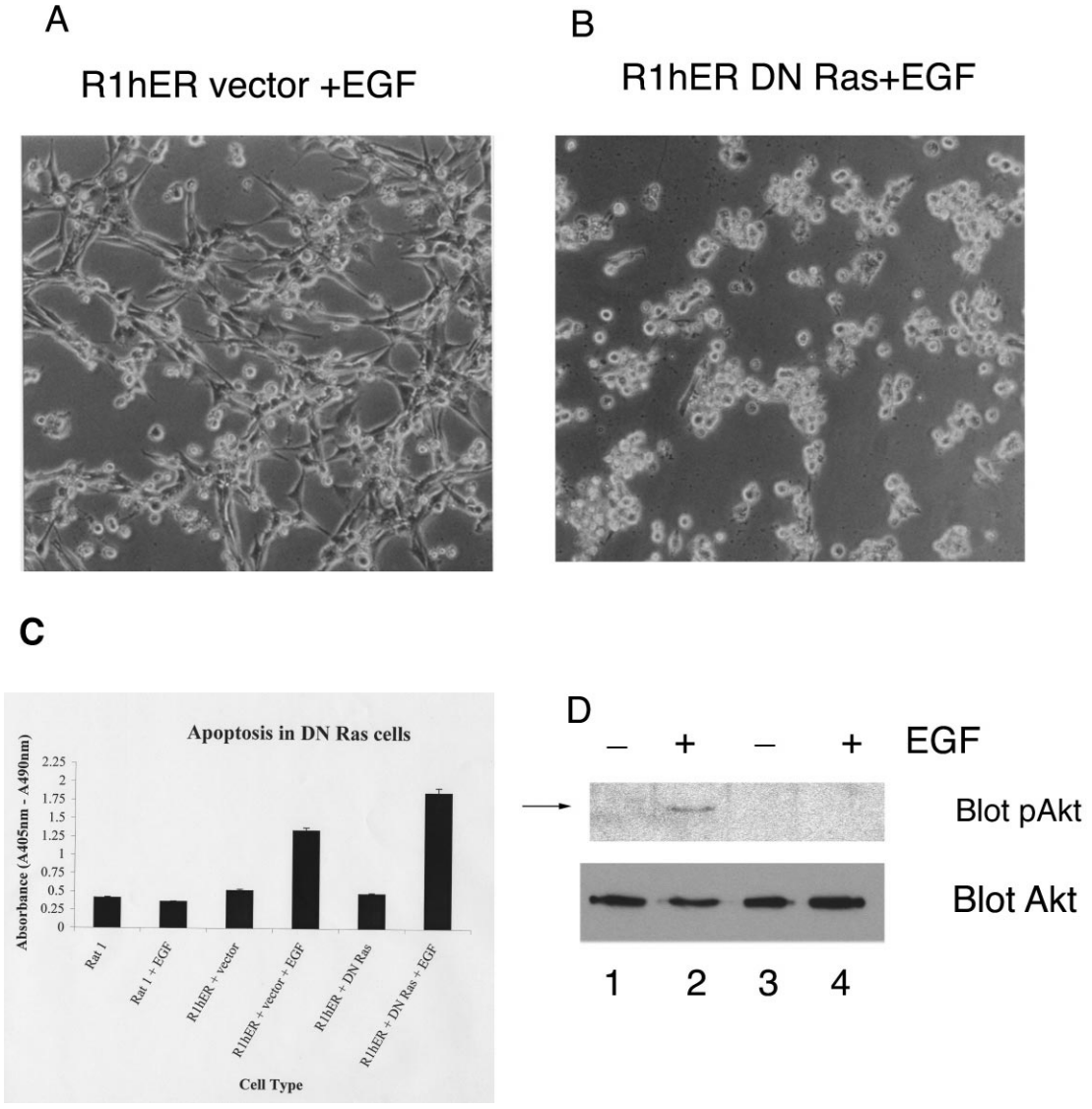

Fig. 5. Expression of a dominant negative Ras mutant potentiates EGF mediated apoptosis in R1hER cells. A: Vector transfected R1hER cells. B: R1hER cells expressing dominant negative Ras after treatment with 5 ng/ml EGF for 48 h. Cells were treated with dexamethasone prior to EGF exposure. An increase in tyrosine phosphorylation of the EGFR can be detected in R1hER cells upon exposure to 5 ng/ml EGF (data not shown). C: Increased apoptosis in DN Ras cells as measured by a cell death detection ELISA. The experiment is representative of three independent experiments done in triplicate. D: Expression of dominant negative Ras in R1hER cells blocks EGF induced Akt activation (lanes 3 and 4) while Akt activation is intact in cells expressing empty vector. Immunoblotting was performed with a phospho-Akt (serine 473 antibody). The blot was stripped and reprobed with Akt antibodies to show protein loading (lower panel).

This suggests that caspase activation may be a late feature of EGFR induced apoptosis.

The EGFR is overexpressed in many types of cancers and correlates with malignant progression. In this study we demonstrate that apoptosis is a predictable outcome of EGFR overexpression in a variety of cell types. To reconcile these apparently conflicting observations, we propose the following hypothesis. During the clonal evolution of a tumor low levels of receptor activation may promote mitogenesis and confer a growth advantage to cells. Since the levels of circulating ligand in vivo are low, tumors may select for cells overexpressing receptors to sustain their growth. Beyond a certain threshold of receptor expression, however, cells may undergo apoptosis as a result of high levels of receptor activation when exposed to exogenous ligand, especially at pharmacologic doses. Whether there are subsets of human tumors overexpressing the wild-type EGFR that undergo apoptosis in response to EGF in vivo remains to be determined, and is, we believe, a question that deserves further study.

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