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Epidermal Growth Factor Protects Fibroblasts from Apoptosis via PI3 kinase and Rac Signaling Pathways

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Abstract

The fibroplasia noted during wound repair is resolved by fibroblast cell death. How fibroblasts undergo death and how this is prevented by trophic growth factors present during the regenerative phase are unknown at the molecular level. We examined a model of staurosporine-induced apoptosis in fibroblasts. We demonstrated that epidermal growth factor (EGF) stimulation of fibroblast NR6WT expressing human EGF receptors blocks staurosporine-induced apoptosis by inhibiting the activation of caspase-3. The survival effect of EGF on rescuing apoptotic NR6WT involves signaling pathways that derive from PI3K and Rac; the blockade of apoptosis is abolished when PI3K and Rac signals are inhibited simultaneously. Furthermore, by using KP372-1, a specific Akt inhibitor, we found that downstream of Akt signaling pathways is absolutely required for the EGF rescue from staurosporine-induced apoptosis in NR6WT. Interestingly, EGF prevention of apoptosis induced by TNF- α in the face of cycloheximide blockade of protein translation occurs via a different set of pathways as the simultaneous inhibition of ERK, Rac and PI3K signaling did not eliminate EGF from rescuing fibroblasts in the face of this cytokine. These findings indicate that EGF receptor activation provides survival response against staurosporine-induced apoptosis through signal pathways of PI3K and Rac which then may prevent the activation of caspase-3.

Keywords

EGF; Fibroblast; Apoptosis; Staurosporine

INTRODUCTION

Wound repair undergoes successive regenerative and resolving phases, of which a striking characteristic is an exuberant cellular ingrowth followed by loss of cells and structures mainly by apoptosis.^{1, 2} While the external signals that induce this hypercellular response and the intracellular pathways responsive for the immigration are being deciphered,^{3, 4} the underpinnings of the cellular loss are less clear. During normal wound healing, granulation tissue becomes replaced coincident with a massive apoptosis of granulation tissue myofibroblasts and vascular cells that had previously accomplished the epithelialization.⁵ This may be due to a reduction in growth factors and/or increase in metalloproteinases at this transition, resulting in the fibroblast and vascular cell apoptosis, as reported during the rapid tissue remodeling induced by a vascularized skin flap.⁶ Molecular signals and

cascades are emerging on the involution of vasculature and death of the endothelial cells. However, the reversion of the fibroplasia is not well studied.

As an initial approach to this question, we examined whether trophic growth factors present during the regenerative fibroplasia could prevent induction of fibroblast apoptosis. Numerous growth factors are released from the initial hemostatic platelet plug and during the immediate but transient inflammatory phase of healing. Prominent among these are ligands for the epidermal growth factor (EGF) receptor (EGFR). TGF- α is a major component of platelet granules and heparin-binding (HB)-EGF is produced in large quantity by macrophages. The levels of these EGFR ligands decline late in the regenerative phase and remain low in the resolving phase. EGFR signaling initiates fibroblast immigration and proliferation.⁷⁻⁸ In addition, as EGFR signaling has been shown to be protective from apoptosis in other cell types,⁹⁻¹¹ it is likely that these ligands also protect fibroblasts from the sea of pro-apoptotic cytokines and other stressors present early in wound repair.

Apoptosis, a programmed cell death which caused by intra- or extracellular inducers, is the main mode of removing cells during physiological involutions of organogenesis and neo-organogenesis (wound repair). While the terminal events of cell rounding, membrane blebbing, cytoskeletal collapse, cell volume shrinking, nuclear pyknosis, chromatin condensation/fragmentation,¹² and the final molecular effector pathway through the caspase cascade¹³ is well-described, the upstream activators and inhibitors are unknown for fibroblasts. In other cells, mainly cancer cells, investigators have found that EGFR signaling pathways involving ERK, PI3K, protein kinase C, and Rac are involved in the protection against apoptosis.⁹⁻¹⁴⁻¹⁶ However, whether similar pathways are operative in fibroblasts to maintain cell viable, such as during wound healing, is not known and is invested in this study. As such, we sought to examine such pathways in vitro, utilizing a nonphysiological though widespread and powerful inducer of cell apoptosis, staurosporine, and whether this cell death is prevented by the presence of a growth factor family that promotes the early stages of wound healing, EGF.

MATERIALS AND METHODS

Cell Lines

Parental NR6 mouse fibroblasts were selected to not express EGF receptor through original Swiss 3T3 cells. NR6WT was generated by transfecting parental NR6 cells with pCEP4-EGFR construct which contains human EGF receptor cDNA. Stable colony was selected in complete growth medium with 200 μ g/ml Hygromycin B. All culture reagents were from CellGro (Herndon, VA). Hs68 normal human diploid fibroblasts were purchased from American Type Culture Collection, and thus were exempted as Human Subjects by the University of Pittsburgh IRB. Hs68 cells were used at passage 5–12 to avoid aging effects.

Antibodies and Other Reagents

Antibodies were from the following sources: phospho-Akt (Ser⁴⁷³), phospho-ERK (Thr²⁰²/Tyr²⁰⁴), Caspase-3, PARP and Stat3 were from Cell Signaling Technology (Beverly, MA). Actin and Rac antibodies were from Sigma. Secondary horseradish peroxidase-conjugated antibodies used for Western blot were purchased from Amersham Biosciences (Piscataway, NJ). PD98059 and LY294002, Akt inhibitor IV were from EMD Calbiochem (San Diego, CA). Hygromycin B was from Invitrogen (Carlsbad, CA).

Plasmids and DNA

Plasmid pCEP4-EGFR was constructed by cloning full length of human EGF receptor cDNA into restriction enzyme sites of Hind III and BamH I. pcDNA3-EGFP-T17NRac was a kind gift from Dr. Gary M. Bokoch (Scripps Research Institute, CA).

Cell culture and Transfection

NR6WT cells were maintained in alpha-MEM supplemented with 7.5% fetal bovine serum, 1× non-essential amino acids, 1× L-glutamine, 1× sodium pyruvate, 1× penicillin/streptomycin and 200 µg/ml Hygromycin B. Transient transfection of NR6WT was performed at 90–100% confluence using Lipofectamine™ 2000 according to the manufacture's recommendations. The total amount of DNA was maintained at 5 µg per 3-cm Petri-dish plate for constructs and 20 nM for GFP and STAT3 siRNAs. The transfection time was between 18 to 24 hours.

Immunoblotting Analysis

After treatments, all cells including floating cells were harvested in 2× cell lysis buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 5% β-Mecapto-ethanol and 0.05% bromophenol blue) and then boiled for 5 min followed by centrifugation at 10,000g for 5 min. Supernatant was loaded to SDS polyacrylamide gel for electrophoresis and then transferred to PVDF membrane. After blocking with a 5% nonfat dried milk TTBS solution for 30 min, the membrane was incubated with primary antibody at optimum dilution at 4°C overnight. Membrane thoroughly washed with TTBS was incubated at room temperature with an appropriate peroxidase-conjugated antibody at 1:4,000 dilution for 45 min. After 3 times washing the antibody binding was detected using ECL reagents.

Apoptosis Evaluation

The cleavage of caspase-3 in apoptotic cells was monitored by immunoblotting detection. Apoptosis in whole cell populations was quantitated by counting apoptotic cells. Following stimulations, cells were stained in situ with 20 µg/ml Hoechst 33342 at 37°C for 20 min following by photography under a fluorescent microscope. Cells with condensed or fragmented nuclei were considered as apoptotic cells.

RESULTS

EGF prevents staurosporine-induced apoptosis

Staurosporine induces apoptosis in many kinds of cells including fibroblasts. We established that staurosporine (50 nM) activates the integrating effector caspase-3 within two hours (Figure 1A). The apoptotic phenotype was visualized by using the dye Hoechst 33342 (Figure 1B). It should be noted that to best isolate the effects of growth factor, these studies were performed in low serum, which induces a low but consistent and accountable level of apoptosis even in the absence of staurosporine induction. This activation of the apoptotic cascade was blocked by EGF (1nM) (Figure 1B-D). Specificity for EGF was shown by both the need for EGFR, the EGFR-devoid NR6 cells were not rescued from staurosporine-induced apoptosis, and prevention of protection by the EGFR inhibitor PD153035 at the concentration of 10 µM (Figure 1D). As shown in Figure 1D, the phosphorylation of ERK and Akt was inhibited by the EGFR-specific agent, PD153035. This suggested that EGFR downstream signaling pathway is essential for EGF effect on rescuing NR6WT from apoptosis induced by staurosporine. Furthermore, in all these experiments the activation of caspase-3 correlated directly with visualization of nuclear fragmentation (and was validated further by Annexin V staining (data not shown)), enabling one method to stand for both in further studies.

EGF rescue from apoptosis occurs via multiple intracellular signaling pathways

A number of pathways from EGFR that have been implicated in cell survival were probed individually to determine whether they are required for the EGF rescue from staurosporine-induced apoptosis (Figure 2). Inhibition of ERK MAP kinase with PD98059 (10 μ M) (Figure 2A) or PI3K with LY294002 (10 μ M) (Figure B) alone did not prevent the EGF rescue. The slight staurosporine induction of ERK activation is not unexpected due to both the biphasic nature of activation/downregulation of PKC and the myriad effects of such inhibition; still this slight increase in pERK is a fraction of that induced by classic growth factor ligands. Interestingly, in both cases the inhibitor appeared to increase slightly the staurosporine-induced activation of caspase-3 and cleavage of its nuclear target PARP. Of note, PI3K inhibition did not fully overcome EGF rescue, suggesting that PI3K pathway contributed to EGF rescue, but was not absolutely required.

A third pathway implicated in survival is that via STAT3 which is activated by EGF (Figure 2C). STAT3 was downregulated by specific siRNA treatment (Figure 2D and 2E). Comparing to GFP siRNA control, EGF still prevented the apoptotic signal in NR6WT transfected with specific STAT3 siRNA (Figure 2E, lane 9). These results suggested that there are other signaling pathways involved in EGF effect on rescuing apoptosis induced by staurosporine.

Rac is a key molecule for EGF rescue

The above suggests that none of the canonical pathways in itself is solely required for rescue from staurosporine-induced apoptosis by EGF. This opens the question of whether elimination of all three pathways together would prevent EGF rescue from apoptosis (Figure 2E, lane 12). Even this triple inhibition did not prevent EGF rescue from apoptosis.

Another candidate pathway is that via the small GTPase Rac that has been implicated in survival. 16 We eliminated Rac signaling using a dominant-negative construct (EGFP-N17Rac) (Figure 3). In itself, this did not activate caspase-3. However, concurrent inhibition of PI3K and negation of Rac, did prevent EGF rescue from staurosporine-induced apoptosis. These findings suggest that both PI3 kinase and Rac pathway contribute to EGF-triggered survival and that at least one of these pathways must be intact. EGF does not rescue staurosporine-induced apoptosis by increasing the anti-apoptotic protein Bcl-xL, as levels stay constant (data not shown) as reported in other systems. 17, 18

Downstream of Akt signaling pathway is absolutely required for EGF rescue from apoptosis

A key signaling switch downstream from both PI3K and Rac is Akt/PKB. In order to determine if the downstream of Akt signaling pathway is required for EGF rescue from apoptosis, we tested the EGF effect on rescuing staurosporine-induced apoptosis in NR6WT in the presence of a specific Akt inhibitor KP372-1 which has been shown to inactivate the kinase activity of Akt. As shown in Figure 4, KP372-1 itself did not induce any apoptosis (lane 4), but it enhanced the apoptosis induced by staurosporine (lane 6). However, EGF failed to rescue apoptotic NR6WT induced by staurosporine when 2.5 μ M KP372-1 was added. The effects of KP372-1 were reproduced by a second inhibitor, AKT inhibitor IV (data not shown). These data suggest that downstream of Akt is required for EGF rescuing staurosporine-induced apoptotic NR6WT.

EGF rescues NR6WT from apoptosis induced by the combination of TNF- α and cycloheximide

During the first few days of wound repair, the immigrating fibroblasts are presented with a number of pro-apoptotic cytokines. It is important for the subsequent regenerative phase that

these cells survive this hostile environment. As such we challenged fibroblasts with a common wound cytokine, tumor necrosis factor- α (TNF- α) (Figure 5). In the face of limited de novo protein translation as inhibited by cycloheximide, TNF- α induced NR6WT apoptosis which could be rescued by concomitant EGF treatment. To determine if this occurred via the same PI3K and Rac signaling pathways, the cells were transfected with either control EGFP DNA construct or the N17-Rac dominant negative construct fused with EGFP and tested in the presence of the PI3K inhibitor LY294002 (and the MEK inhibitor PD98059). This triple inhibition failed to prevent EGF-triggered rescue from apoptosis (lane 18), suggesting even additional pathways, not dependent on protein translation, by which EGFR signaling maintains the viability of fibroblasts.

DISCUSSION

Programmed cell death is recognized as a critical physiological part of organogenesis. This also occurs late in wound repair to remove excess cellularity that is part of the regenerative phase of wound repair. Currently, there is little known about the mechanisms by which this happens for fibroblasts, 3 cells that form the bulk of the dermis and present a significant hyperplasia during the early stages of repair. 2· 19· 20 As it is an axiom in regulatory biology that cellular events are affected by a balance between inductive and counter-inductive signals, we examined intracellular signals leading to fibroblast death in the absence of the survival-promoting growth factors that are present during the regenerative phase. Ligands for EGFR are prominently present during the earlier fibroplasia stage but are significantly reduced, coincident with loss of fibroblasts during the resolving phase. 1 In the absence of EGFR ligands, we find that fibroblast apoptosis can be induced by standard inducers including both a pharmacological agent, staurosporine, and a cytokine, TNF- α . Growth factor-signaled rescue from apoptosis is multifaceted as numerous intracellular signaling pathways prevent apoptosis. At least two pathways, via Rac and PI3K, are each individually capable of negating staurosporine-induced apoptosis, with TNF- α -induced apoptosis being circumvented by at least three pathways, as dual blockage of Rac and PI3K did not prevent EGF-induced rescue. This suggested a complex machinery designed to preserve fibroblasts in the face of a changing and cytokine-rich wound milieu.

Our data point towards multiple pathways downstream of EGFR that mediate fibroblast survival even in the face of apoptosis inducers. This is likely relevant to the situation in wounds in that ligands for growth factor receptor, which are at high levels early on in wound healing, drop as the repair process proceeds to resolution. 1 We do not, however, know the nature of the physiological inducers of apoptosis in this realm; as such, we cannot comment on whether these increase or decrease at these later stages change the balance between survival and death signals. We did test a direct, but not physiological, inducer of apoptosis in staurosporine, a well known inhibitor of cellular protein kinases. 21 This allowed the dissection of the survival signals in the presence of strong death signals. Secondly, we tested survival pathways in the presence of a physiological constituent of wounds that may signal fibroblast death, TNF- α . This cytokine does not drive fibroblast apoptosis in itself, but only in the presence of an inhibitor of protein expression (cycloheximide). These two agents are commonly used in combination to stress cells into apoptosis, 22· 23 presumably CHX is generally thought to block NF- κ B activation, which is simultaneously induced by TNF- α . The TNF- α /CHX exposure in itself activated the ERK and p38 but not the JNK MAP kinase pathways (data not shown) similarly to staurosporine, though whether this helps promote the apoptosis or is a reflective counter response requires further study. This is not likely critical to the effects on EGF as these signal activations are not reverted by EGF rescue of TNF- α /CHX apoptosis.

At first it may seem daunting that multiple pathways from a single growth factor can signal independently cell survival, overcoming strong apoptotic inducers. This suggests that, at least for fibroblasts, survival is a dominant phenotype. It is assumed that these responses would also relate to myofibroblasts, as the NR6 WT cells used herein express smooth muscle actin. Further, these challenges also induced apoptosis, with at least partial rescue by EGF, in the human primary dermal fibroblast Hs68, which also expresses alpha smooth muscle actin.

In the skin, which is subject to repeated injury and localized infections with the production of numerous pro-apoptotic cytokines, this resistance to death cues would provide for tissue homeostasis. As the survival signaling identified, via PI3K and Rac and possibly others including ERK and STATs, actuate a number of cellular processes, this redundancy allows for modulation of other cellular events without driving the cells into an apoptotic mode. For instance, attenuation of Rac activity, being involved in lamellipod extension during motility 24 may be critical for dermal fibroblast maturation; however, the conversion from a motile cell to a synthetic sessile fibroblast, a critical step in the resolving wound dermis, would not be accomplished if the cell was now susceptible to killing signals. Deciphering the ramifying network of survival and death signals in fibroblasts will likely shed light also on pathways that lead to other cell processes.

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Abbreviations

EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
PI3K	phosphatidylinositol-3'-kinase
TNF-α	tumor necrosis factor-alpha

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Figure 1.

EGF protects NR6WT from staurosporine-induced apoptosis: (A) Time course of staurosporine-induced apoptosis in fibroblasts NR6 cells (upper panel) and NR6WT (lower

panel). Cells quiesced with quiescence media in the presence of 0.1% fetal bovine serum for 18h were treated with 50 nM staurosporine for the indicated time, and then all cells were collected, lysed and immunoblotted for caspase-3. The increase in caspase 3 over time was statistically significant compares to NT (time 0). (B) Nuclear staining of fibroblasts treated with staurosporine in the presence or absence of EGF for 3h in NR6 (upper, devoid of EGFR) and NR6WT (lower, which express EGFR). After treatment, cells were incubated with 20 ug/ml Hoechst 33342 for additional 20 min followed by imaging under fluorescent microscope. The percent of apoptosis was determined by counting at least 300 cells in three randomly chosen fields for each treatment. The EGF protection was significant between these two cell types. (C, D) Cells treated with staurosporine with or without EGF and EGFR inhibitor PD153035 were collected and lysed for immunoblotting against caspase-3, pERK and pAkt. In (D) only NR6WT cells are shown. In all parts, results are representative, or means $\pm$ s.d. of at least three independent experiments. The amount of cleaved caspase-3 has been quantitated using the software NIH Image J. NT, non-treated, ST, staurosporine.

Figure 2.

Identification of signaling pathways involved in EGF function on rescuing NR6WT from staurosporine-induced apoptosis: (A, B) Quiesced NR6WT cells were incubated with the indicated reagents for 3h. All cells were collected, lysed and immunoblotted for caspase-3 and pERK, PARP, pERK and pAkt. There were significant differences between ST-driven apoptosis and EGF rescue othis regardless of inhibitor (PD98059 or LY294002) treatment. (C) NR6 and NR6WT cells quiesced for 18h were treated with 1 nM EGF for the indicated time and then all cells were collected, lysed and immunoblotted for total STAT3 and phosphor-STAT3. (D) Knockdown of STAT3 by siRNA transfection. NR6WT were transfected with STAT3 siRNA for either 24 or 40h and then all cells were harvested, lysed and immunoblotted for STAT3. (E) NR6WT transfected with STAT3 siRNA for 40h and quiesced for 18h were treated with the indicated reagents for 3h. Then all cells were collected, lysed and immunoblotted for caspase-3, pERK, pAkt and total STAT3. There is no difference in caspase cleavage regardless of STAT3 siRNA downregulation. In all sections, the amount of cleaved caspase-3 has been quantitated using the software NIH

Image J. NT, non-treated; ST, staurosporine; PD, PD98059; LY, LY294002. Results are representative of at least 3 separate experiments.

Figure 3.

Inhibition of Rac and PI3K signaling pathways prevents EGF rescuing NR6WT from staurosporine-induced apoptosis: NR6WT transfected with either control EGFP DNA construct or plasmid DNA which expresses dominant negative N17Rac fused by EGFP for 24h were quiesced for 18h. Starved cells were treated with indicated reagents for 3h and then all cells were collected, lysed and immunoblotted for caspase-3 and actin. The amount of cleaved caspase-3 has been quantitated using the software NIH Image J. There is significant diminution of EGF rescue in the presence of the dominant negative N17Rac regardless of treatment. NT, non-treated; ST, staurosporine; PD, PD98059; LY, LY294002. Results are representative of 3 experiments.

Figure 4.

Inhibition of Akt kinase activity prevent EGF rescuing NR6WT: NR6WT quiesced with quiescence medium in the presence of 0.1% fetal bovine serum for 18h were pretreated with 2.5 uM KP372-1 for 15 min and then further treated with indicated reagents for 3h. All cells were harvested, lysed and immunoblotted for caspase-3, phospho-Akt (ser 473), total Akt, and actin. The amount of cleaved caspase-3 has been quantitated using the software NIH Image J. NT, non-treated, ST, staurosporine. Similar results were obtained with an alternative Akt inhibitor, Akt inhibitor IV (data not shown).

Figure 5.

EGF rescues NR6WT from apoptosis induced by the combination of TNF- α and cycloheximide: NR6WT transfected by either control EGFP DNA construct or plasmid EGFP-N17Rac were quiesced with quiescence medium containing 0.1% fetal bovine serum for 18h. Then cells were pre-treated with 10 μ M cycloheximide for 20min and were further treated with 10 ng/ml TNF- α , 1 nM EGF and other indicated reagents for another 3h. All cells were collected, lysed and immunoblotted for caspase-3. The amount of cleaved caspase-3 has been quantitated using the software NIH Image J. There is no significant difference in cell responses depending on the presence or absence of the dominant negative N17Rac. NT, non-treated; TC, TNF- α + cycloheximide; PD, PD98059; LY, LY294002. Results are representative of 3 experiments.

Table 1

Antibody target	Vendor	Host	Phosphorylation site
Caspase-3	Cell signaling Technology	Rabbit	Thr ²⁰² /Tyr ²⁰⁴
pERK	Cell signaling Technology	Rabbit	Ser ⁴⁷³
pAkt	Cell signaling Technology	Mouse	
Actin	Sigma	Rabbit	
Stat3	Cell signaling Technology	Rabbit	
PARP	Cell signaling Technology	Rabbit	
Rac	Sigma	Mouse	
Inhibitor name	Vendor	Concentration	Function
PD98059	EMD Calbiochem	10 uM	pERK inhibitor
LY294002	EMD Calbiochem	10 uM	PI3K inhibitor
PD153035	EMD Calbiochem	10 uM	pEGFR inhibitor
KP372-1	Echelon	2.5 uM	Akt inhibitor
Akt inhibitor IV	EMD Calbiochem	2.5 uM	Akt inhibitor