Heparin-binding epidermal-growth-factor-like growth factor gene expression is induced by scrape-wounding epithelial cell monolayers: involvement of mitogen-activated protein kinase cascades

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Peptide growth factors can promote the cell migration and proliferation that is needed to repair epithelia after mechanical or chemical injury. We report here that scrape-wounding rat intestinal epithelial (RIE-1) cell monolayers caused a rapid increase in levels of heparin-binding epidermal-growth-factor-like growth factor (HB-EGF) mRNA, with a maximal response at approx. 1 h. Hybridization *in situ* showed that transcript induction occurred primarily in cells at or near wound borders. The increase in HB-EGF mRNA was preceded by activation of the p42 mitogen-activated protein kinase (MAPK) in the wounded cell cultures. Moreover, the induction of HB-EGF mRNA was blocked by PD098059 and U0126, inhibitors that prevent the activation of p42/p44 MAPKs and extracellular signal-regulated protein kinase 5 (ERK5). Both p42 MAPK activation and HB-EGF mRNA induction were inhibited by genistein, indicating a requirement for an upstream tyrosine kinase activity. In contrast, neither response was affected by inhibition of phosphoinositide 3-kinase activity, down-regulation of protein kinase C, or disruption of the actin cytoskeleton with cytochalasin B. We conclude that scrape-wounding epithelial cell monolayers induces HB-EGF mRNA expression by a mechanism that most probably requires p42/p44 MAPK activation, although we cannot exclude a role for ERK5. Our results suggest a physiological role for locally synthesized HB-EGF in promoting epithelial repair after injury.

Key words: epithelial repair, phosphatidylinositol 3-kinase, protein kinase C, protein tyrosyl kinase.

INTRODUCTION

Heparin-binding epidermal-growth-factor-like growth factor (HB-EGF) is a member of the family of factors that includes epidermal growth factor (EGF), transforming growth factor α, amphiregulin, betacellulin and epiregulin. HB-EGF was originally found in conditioned medium from cultured macrophages and the U937 macrophage-like cell line [1–3]. HB-EGF released by U937 cells comprises several heparin-binding proteins of 19–23 kDa [3]. Subsequent studies have demonstrated that these soluble forms of HB-EGF are derived from the processing of a plasma membrane-anchored 'precursor' protein [2,4]. The mitogenic activity of soluble HB-EGF was originally demonstrated with smooth-muscle cells and fibroblasts [1–3] but other cell types including hepatocytes, keratinocytes, kidney tubule cells and gastrointestinal epithelial cell lines also respond to the factor [5].

Northern blot and PCR analyses indicate that the HB-EGF gene is widely expressed [6,7]. Although HB-EGF mRNA levels seem to be relatively low in normal tissues, expression increases in response to tissue damage. For example, HB-EGF mRNA levels rise rapidly in lungs in response to hyperoxia [8], in kidneys subjected to ischaemia/reperfusion injury [9] and in liver after partial hepatectomy or chemical damage [10,11]. Furthermore, HB-EGF is present in skin wound fluids [12,13]. Inducible expression of HB-EGF mRNA has also been observed *in vitro*; in various cell types, soluble agonists including serum, hormones and growth factors have been shown to stimulate large, generally transient, increases in HB-EGF mRNA levels [5,14]. Although the mechanisms involved in activating HB-EGF gene transcription are not fully understood, the Raf–MEK [mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase kinase]–MAPK pathway seems to have an important role [15–17].

It has recently been reported that scrape-wounding epithelial cell cultures causes localized activation of the p42/p44 MAPK pathway [18,19], with activation extending several cell-lengths from the wound border [18]. Taken together, these results raise the possibility that denuding injury to epithelial cell monolayers could activate HB-EGF gene expression. We have tested this hypothesis with a non-transformed epithelial cell line (RIE-1) derived from rat intestine, in which HB-EGF mRNA levels are strongly induced by soluble agonists [20]. Here we demonstrate that HB-EGF mRNA levels increase rapidly in scrape-wounded RIE-1 cell monolayers and that this induction is prevented by inhibitors of MEK/MAPK activation. Because HB-EGF stimulates the survival [21], proliferation [20] and migration [22] of epithelial cells, our results suggest a physiological role for induced HB-EGF expression in promoting localized epithelial repair after injury.

EXPERIMENTAL

Reagents

Radioisotopes were obtained from Amersham Pharmacia Biotech or ICN. The anti-(p42 MAPK) monoclonal antibody was from Transduction Laboratories. Anti-(phospho-protein kinase B), anti-(protein kinase B) and anti-(phospho-p38 MAPK) antibodies were from New England Biolabs. PD098059, U0126,

Abbreviations used: EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; GAPDH, glyceraldehyde-phosphate dehydrogenase; HB-EGF, heparin-binding EGF-like growth factor; MAPK, mitogen-activated protein kinase; MEK, MAP kinase/ERK kinase; PKB, protein kinase B; PKC, protein kinase C; PI-3K, phosphoinositide 3-kinase.

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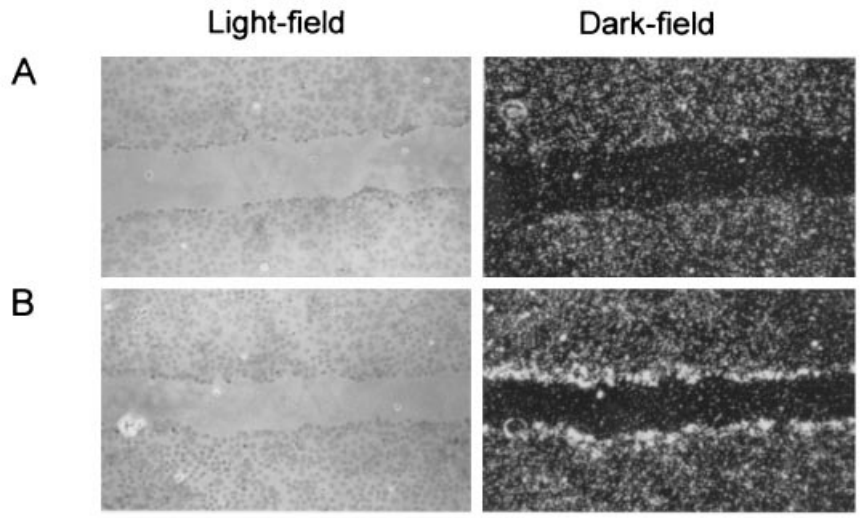

Figure 1 Localization of HB-EGF mRNA induction in wounded cell monolayers

Confluent monolayers of RIE-1 cells were scrape-wounded with a plastic pipette tip and fixed either immediately (**A**) or 90 min after wounding (**B**). Fixed cells were processed for hybridization *in situ* with ^{35}S-labelled HB-EGF anti-sense riboprobes as described in the Experimental section.

SB203580, LY294002 and wortmannin were from Calbiochem. Ro31-8220 was purchased from Calbiochem or Alexis Biochemicals.

Cell culture

RIE-1 cells were grown on plastic culture dishes (Nunc) in Dulbecco's modified Eagle's medium containing 5% (v/v) newborn calf serum, 100 i.u./ml penicillin and 100 μg/ml streptomycin.

Hybridization *in situ*

Cells were seeded on gelatin-coated glass coverslips (22 mm × 55 mm) and grown to confluence. Cell monolayers were wounded by scraping a sterile plastic pipette tip in a straight line across the length of the coverslip. The coverslips were placed on ice, rinsed twice with ice-cold PBS and fixed for 15 min with PBS containing 4% (w/v) paraformaldehyde at 4 °C. After fixation, cells were rinsed twice with PBS, twice with 0.85% NaCl solution, both at room temperature, and dehydrated through an ascending ethanol series. The coverslips were processed for hybridization *in situ* as described [23]. Uridine 5′-[[α-^{35}S]thio]triphosphate-labelled sense and anti-sense probes were generated from a PCR-derived fragment spanning nt 23–667 of rat HB-EGF cDNA [7] cloned in Bluescript M13$^+$. Cells were hybridized overnight at 52 °C with the radiolabelled probes and washed at high stringency, including a 30 min wash in buffer containing ribonuclease A (5 μg/ml). Coverslips were coated with photographic emulsion (Ilford K5). After 3 weeks at 4 °C the emulsion was developed and the cells were stained with haematoxylin.

Scrape-wounding of cell monolayers

Multiple wounds were made with a 4 cm portion of a standard plastic hair comb. Dishes (9 cm in diameter) of cells were placed on the bench, and starting from the far end of the dish, two parallel, non-overlapping scrapes were made to the opposite side of the dish. The dish was then rotated and scrapes were made as above at 45°, 90° and 135° to the original scrapes. As controls, cultures were mock-wounded by moving the comb as described above through the medium, but without damaging the cell monolayer.

p42 MAPK mobility-shift assays

The activation of p42 MAPK can be determined by the decreased mobility of the protein on SDS/PAGE that occurs on formation of the phosphorylated form. Cells were placed on ice, rinsed twice with ice-cold PBS, and solubilized with SDS/PAGE sample buffer [80 mM Tris/HCl (pH 6.8)/2% (w/v) SDS/10% (v/v) glycerol/5% (v/v) 2-mercaptoethanol]. Aliquots of the cell lysates were subjected to SDS/PAGE with a 10% (w/v) resolving gel and a decreased proportion of bisacrylamide (0.16%), which improves the electrophoretic resolution of the phosphorylated and non-phosphorylated forms of p42 MAPK. To maintain approximately equal protein levels, the volume of sample loaded from the wounded cultures was double that loaded for control or mock-wounded cultures. After electrophoresis, proteins were transferred to Immobilon-P membranes (Millipore). Membranes were blocked in PBS containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat milk powder and incubated overnight at 4 °C in this buffer containing 0.06 μg/ml anti-(p42 MAPK) antibody. Immunoreactive proteins were detected with a 1:2000 dilution of sheep anti-mouse Ig conjugated with horseradish peroxidase and the enhanced chemiluminescence (ECL®) detection system (Amersham Pharmacia Biotech) in accordance with the manufacturer's instructions.

Western blot analysis of p38 MAPK and protein kinase B (PKB) phosphorylation

Cells were solubilized with SDS/PAGE sample buffer and aliquots of the cell lysates were subjected to SDS/PAGE [10% or 12.5% (w/v) resolving gel]. To maintain approximately equal protein levels, the volume of sample loaded from the wounded cultures was double that loaded for control or mock-

wounded cultures. Gels were subjected to Western blot analysis as described above with anti-[phospho-(Ser-473) PKB] or anti-[phospho-(Thr-180/Tyr-182) p38 MAPK] antibody solution (New England Biolabs), each at 1:1000 dilution. Immunoreactive proteins were detected with anti-rabbit Ig conjugated with horseradish peroxidase and enhanced chemiluminescence.

Northern blotting

A rat HB-EGF cDNA spanning nt 371–1550 of rat HB-EGF [7] was generated by reverse-transcriptase-mediated PCR and cloned into plasmid Bluescribe M13$^+$. The cDNA was radiolabelled with the random priming method [24]. Cellular RNA isolation, electrophoresis and Northern blotting (10 μg of total RNA per lane) were performed as described [25]. After analysis of HB-EGF mRNA levels, blots were rehybridized with a 250 bp *Hin*dIII–*Pst*I fragment of a glyceraldehyde-phosphate dehydrogenase (GAPDH) cDNA as an indicator of RNA loading.

RESULTS

Denuding injury to epithelial cell monolayers increases HB-EGF mRNA levels

RIE-1 cell monolayers, grown on glass coverslips, were scrape-wounded with a sterile plastic pipette tip. Hybridization *in situ* with a ^{35}S-labelled HB-EGF anti-sense riboprobe revealed increased levels of HB-EGF mRNA in cells along the wound edge, frequently extending three or four cell diameters into the cell monolayer (Figure 1). The hybridization signal was detected in cells that had been incubated for 90 min after wounding but not in control cells that had been fixed immediately after wounding (Figure 1). Furthermore, hybridization with ^{35}S-labelled HB-EGF sense probes did not show an induced signal at wound edges (results not shown).

To study the signalling mechanisms involved in HB-EGF mRNA induction by denuding injury, a plastic comb was used to create multiple wounds simply and reproducibly in RIE-1 monolayers grown on plastic culture dishes (see the Experimental section). Figure 2(A) shows a monolayer that was fixed and stained after scrape-wounding with this procedure. At selected times after wounding, the cells remaining on the dish were solubilized either for RNA isolation and Northern blot measurement of HB-EGF mRNA, or for Western blot analysis of p42 MAPK activation. The results in Figure 2(B) show that HB-EGF mRNA levels increased rapidly in the multiply wounded monolayers, with a maximal response at 1 h. Multiple scrape-wounding also activated p42 MAPK, as demonstrated by a shift of the protein to a lower electrophoretic mobility (Figure 2C), an effect previously correlated with its activation by phosphorylation on tyrosine and threonine [26,27]. Phosphorylation of the kinase was detected as early as 1 min, was maximal at 5–10 min and returned towards basal levels by 60 min (Figure 2B, and results not shown). This time course of activation is similar to those observed when the cells were treated with serum or soluble agonists (results not shown). The shift to phosphorylated pp42 in scrape-wounded cultures was partial, with a proportion of the p42 remaining in the non-phosphorylated form. This partial shift suggests that, even after extensive multiple wounding, p42 MAPK is not activated in a significant fraction of the cells remaining on the dish.

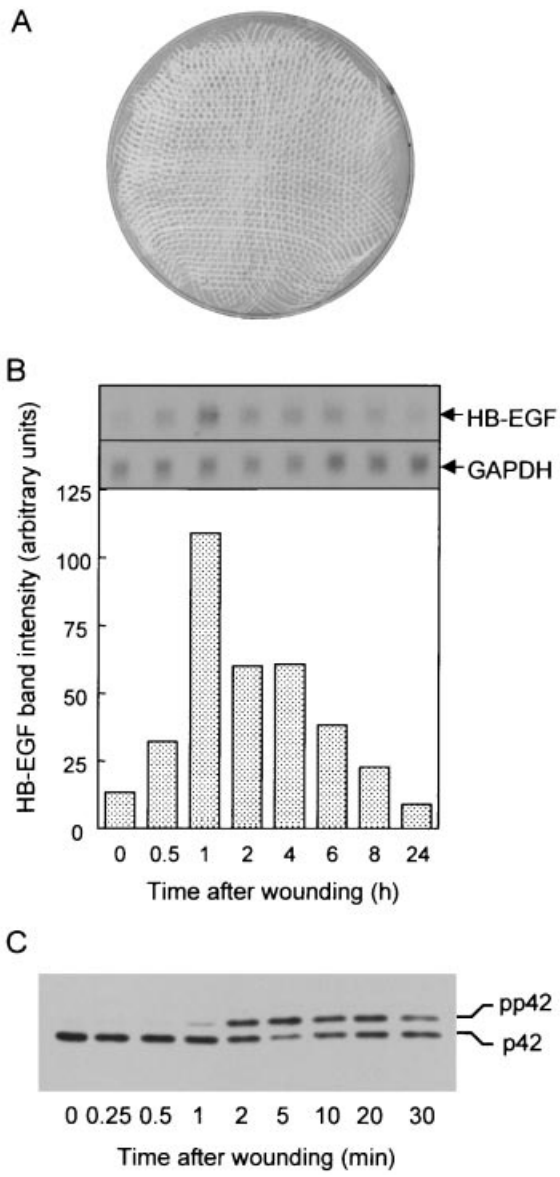

Figure 2 Time course of HB-EGF mRNA induction and p42 MAPK activation in scrape-wounded cell monolayers

(**A**) A monolayer of RIE-1 cells that was fixed and stained after scrape-wounding with a plastic comb as described in the Experimental section. (**B**) Cell monolayers were scrape-wounded and incubated at 37 °C for the indicated durations. After the cells had been rinsed with PBS, total RNA was isolated and aliquots (10 μg) were analysed by Northern blotting with ^{32}P-labelled HB-EGF and GAPDH cDNA species. Autoradiographs were scanned with a Chromoscan 3 densitometer (Joyce-Loebl). The graph shows HB-EGF mRNA levels after correction for minor differences in RNA loading as revealed by the scan of the GAPDH autoradiograph. (**C**) Cell monolayers were scrape-wounded and incubated at 37 °C for the indicated durations. After being rinsed with PBS, the cells were lysed in SDS/PAGE sample buffer. Lysates were analysed by Western blotting with a p42 MAPK antibody. The unphosphorylated (p42) and phosphorylated (pp42) MAPK bands are indicated.

HB-EGF mRNA induction is blocked by MEK inhibitors

To investigate whether there is a causal relationship between the rapid activation of p42 MAPK and the subsequent increase in HB-EGF mRNA levels in the denuded monolayers, we wounded cells in the presence of the MEK inhibitor PD098059. This compound inhibited both wound-induced p42 MAPK phosphorylation and HB-EGF mRNA induction in a dose-dependent manner with a half-maximal inhibition at 3–10 μM (Figures 3A and 3B). A second MEK inhibitor, U0126, was also effective in blocking these responses (Figure 3C).

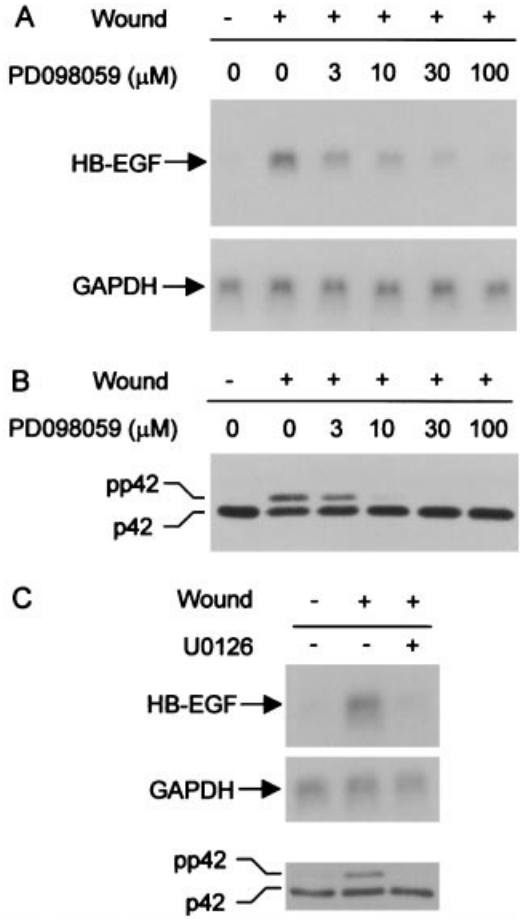

Figure 3 MEK inhibitors prevent HB-EGF mRNA induction by scrape wounding

(**A**, **B**) Confluent monolayers of RIE-1 cells were treated with the indicated concentrations of PD098059 or with vehicle for 1 h. The cultures were scrape-wounded, incubated at 37 °C for 1 h (**A**) or 5 min (**B**) and processed for Northern or Western blot analysis as described in the legend to Figure 2. (**C**) Confluent monolayers of cells were treated with U0126 (20 μM) or vehicle for 30 min. Cultures were wounded or mock-wounded as indicated. The cells were incubated at 37 °C for 60 min before RNA isolation for Northern blot analysis of HB-EGF mRNA or for 5 min before cell lysis for Western blot analysis of p42 MAPK.

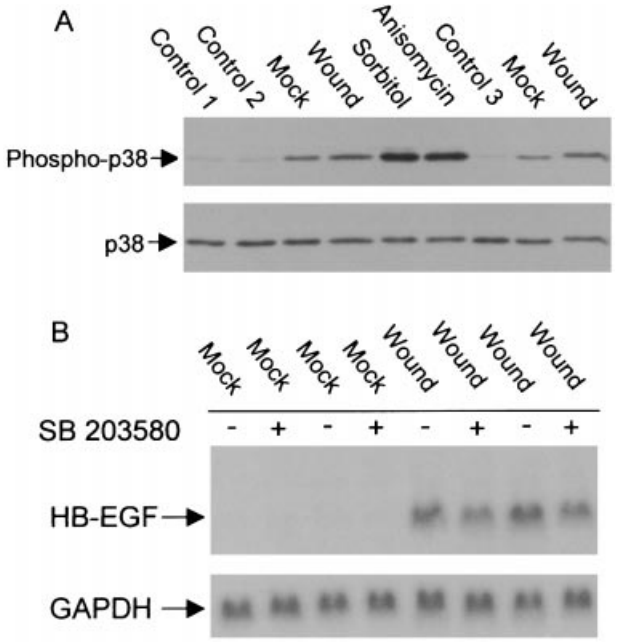

Figure 4 Role of p38 MAPK in HB-EGF mRNA induction by scrape-wounding

(**A**) Confluent monolayers of RIE-1 cells were wounded, mock-wounded, or treated with sorbitol (600 mM) or anisomycin (10 μg/ml). Control cultures received no addition (control 1), DMSO as a vehicle control for anisomycin treatment (control 2) or Dulbecco's modified Eagle's medium as a vehicle control for sorbitol treatment (control 3). The cells were incubated at 37 °C for 15 min, rinsed with PBS and lysed in SDS/PAGE sample buffer. Lysates were analysed by Western blotting with an anti-(phospho-p38 MAPK) antibody. After detection with enhanced chemiluminescence, the blot was reprobed with an anti-p38 antiserum. (**B**) Confluent cell monolayers were treated for 60 min with SB 203580 (10 μM) or vehicle. Cultures were wounded or mock-wounded as indicated. The cells were incubated at 37 °C for 60 min before isolation of RNA for Northern blot analysis of HB-EGF mRNA.

The rapid activation of p42 MAPK in wounded cell monolayers, together with the marked inhibitory effects of PD098059 and U0126, suggest that p42 MAPK activation is required for HB-EGF mRNA induction. It has been reported that a second class of the MAPK family, the p38 stress-activated protein kinases (p38 MAPK), is activated after the wounding of epithelial [18] or endothelial [28] cell monolayers. In RIE-1 cells, mock wounding was sufficient to elicit an increase in the phosphorylation of p38 MAPK in comparison with control cultures, indicating activation of the kinase (Figure 4A). However, wounding the cell monolayers did not lead to a further increase in phospho-p38 levels in comparison with the mock-wounded cultures. Moreover, the phosphorylation of p38 observed in response to mock wounding or wounding was substantially less than that observed in parallel cell cultures that were either treated with anisomycin or subjected to hypertonic stress (Figure 4A). To test the involvement of p38 MAPK activity in wound-induced HB-EGF mRNA expression, we wounded cell monolayers in the presence of SB203580, a selective inhibitor of p38 MAPK. The drug caused a decrease of 20–30 % in the levels of HB-EGF mRNA in the wounded cultures (Figure 4B). This effect is small compared with the effects of the MEK inhibitors, which block HB-EGF mRNA induction (Figure 3C), and suggests that p38 MAPK activity does not play a major part in the wound-induced expression of HB-EGF mRNA.

Roles of protein kinase C (PKC) and phosphoinositide 3-kinase (PI-3K) in HB-EGF mRNA induction

HB-EGF mRNA can be induced in RIE-1 cells through PKC-dependent or PKC-independent pathways (results not shown). To test the role of PKC in wound-mediated activation, we pretreated cells overnight with PMA to down-regulate phorbol-ester-sensitive PKC isoforms. Pretreatment with PMA did not prevent either p42 phosphorylation (Figure 5B) or HB-EGF mRNA induction (Figure 5A) in response to wounding. Because several isoforms of PKC are not down-regulated by pretreatment with phorbol ester, we further investigated the role of PKC with Ro31-8220, a bisindoylmaleimide that is a potent inhibitor of multiple PKC isoforms [29]. Pretreating cells with Ro31-8220 prevented the increase in HB-EGF mRNA levels that occurs after monolayer wounding (Figure 5C). In contrast with this

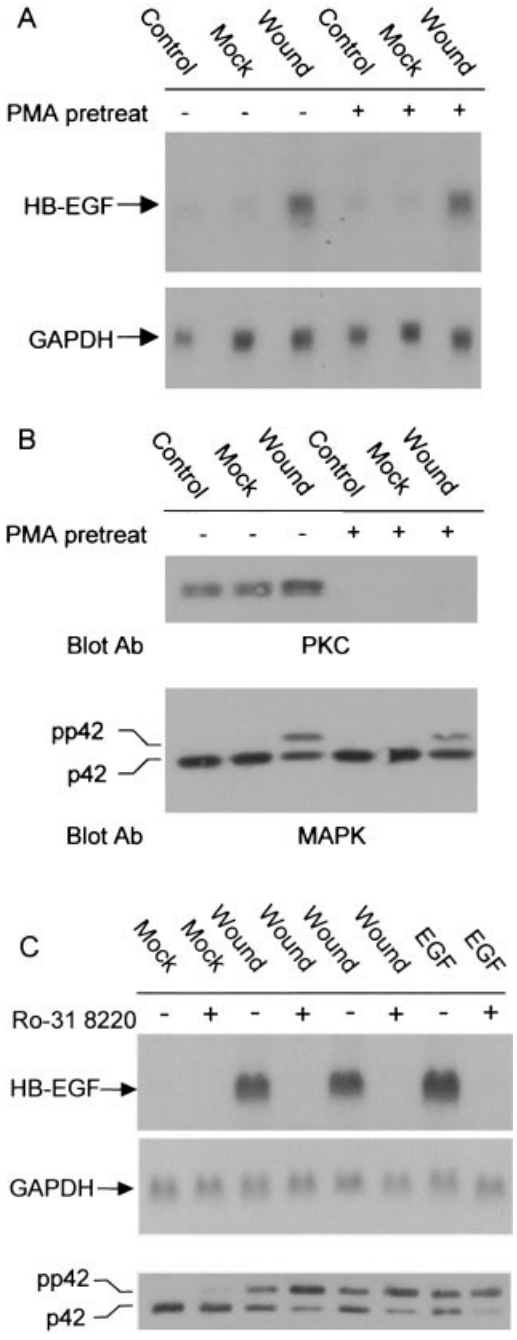

Figure 5 Role of PKC in HB-EGF mRNA induction and MAPK activation by scrape-wounding

(**A**, **B**) Confluent monolayers of RIE-1 cells were treated with PMA (500 nM) for 18 h to down-regulate cellular PKC. Control cultures were treated for the same duration with an equal volume of vehicle. Cultures were wounded or mock-wounded as described in the Experimental section, incubated at 37 °C for 1 h (**A**) or 10 min (**B**), and processed for Northern or Western blot analysis as described in the legend to Figure 2. After incubation in blocking solution, the protein blot was divided into two. To confirm the down-regulation of PKC by pretreatment with PMA, the upper portion was probed with a 1:2000 dilution of a rabbit antiserum raised against the 15 C-terminal amino acids of rat PKC-α. The lower portion of the blot was probed with the p42 monoclonal antibody. (**C**) Confluent monolayers of cells were treated for 60 min with Ro31-8220 (5 μM) or an equal volume of vehicle. Cultures were wounded, mock-wounded or treated with EGF (200 ng/ml) as indicated. The cells were incubated at 37 °C either for 60 min before RNA isolation for Northern blot analysis of HB-EGF mRNA or for 10 min before cell lysis for Western blot analysis of p42 MAPK.

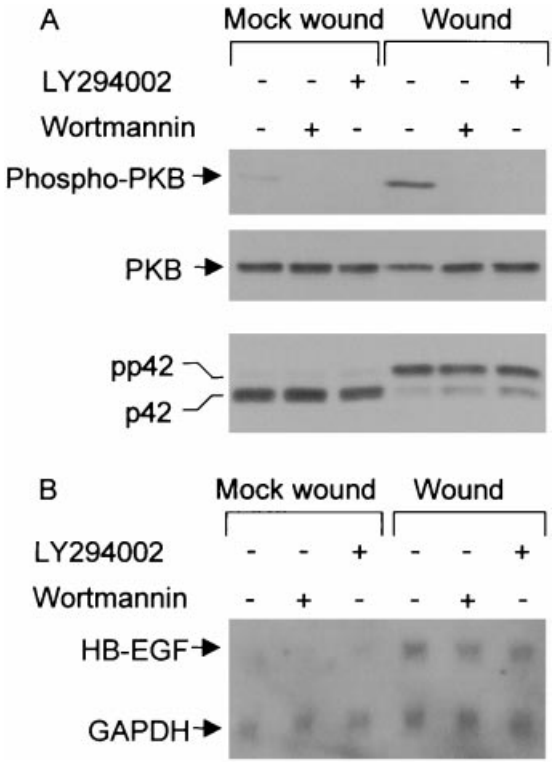

Figure 6 MAPK activation and HB-EGF mRNA induction are independent of PI-3K

Confluent monolayers of RIE-1 cells were treated with wortmannin (100 nM), LY294002 (30 μM) or vehicle for 1 h. (**A**) Cultures were wounded or mock-wounded, incubated at 37 °C for 10 min and lysed in SDS/PAGE sample buffer. Aliquots of cell lysates were resolved by SDS/PAGE and analysed by immunoblotting with anti-(phospho-PKB), anti-PKB or anti-(p42 MAPK) antibodies as described in the Experimental section. (**B**) Cultures were wounded or mock-wounded and incubated at 37 °C for 1 h before cellular RNA isolation and Northern blot analysis. The blot was probed simultaneously with ^{32}P-labelled HB-EGF and GAPDH cDNA species.

marked inhibitory effect, the drug was found to potentiate p42 MAPK activation in response to wounding (Figure 5C). Interestingly, the drug elicited similar effects in EGF-treated cultures; p42 MAPK activation was potentiated, whereas HB-EGF mRNA induction was blocked (Figure 5C). The interpretation of these results is complicated by recent findings that Ro31-8220, in addition to its well-established inhibitory effect on PKC activity, has inhibitory [30,31] and stimulatory [32] actions on other protein kinases (see the Discussion section).

It has been demonstrated that the activation of p42/p44 MAPKs by soluble agonists might require PI-3K activity, particularly at low signal strengths [33,34]. We have found that scrape-wounding activates PI-3K, as indicated by the wortmannin-sensitive phosphorylation of protein kinase B (Figure 6A). To our knowledge, this is the first demonstration of the activation of this pathway by monolayer wounding. However, p42 MAPK activation and HB-EGF mRNA induction by scrape-wounding were unaffected by wortmannin or LY294002 at concentrations that block protein kinase B phosphorylation (Figure 6). We conclude that p42 MAPK activation and HB-EGF mRNA induction in scrape-wounded cell monolayers are independent of PI-3K.

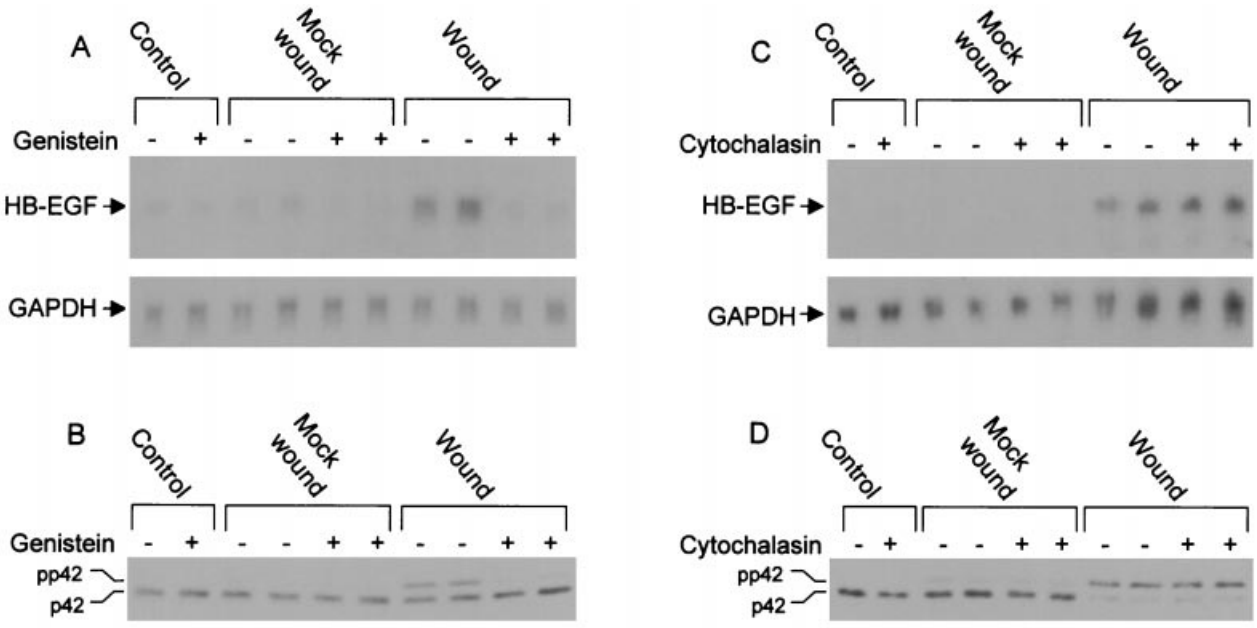

Figure 7 MAPK activation and HB-EGF mRNA induction are inhibited by genistein but not by cytochalasin B

Confluent monolayers of RIE-1 cells were treated for 1 h with genistein (100 μM), cytochalasin B (1 μM) or vehicle. Cultures were wounded or mock-wounded as described in the Experimental section, incubated at 37 °C for 1 h (**A**, **C**) or 10 min (**B**, **D**) and processed for Northern or Western blot analysis as described in the legend to Figure 2.

Protein tyrosine kinase activity is required for HB-EGF mRNA induction by monolayer wounding

Fluid shear stress is a mechanical stimulus that activates p42 MAPK in vascular endothelial cells [35]. This activation is blocked by genistein, indicating the involvement of an upstream protein tyrosine kinase, and by cytochalasin, demonstrating a requirement for an intact actin cytoskeleton [35]. We tested the effects of these drugs on wound-mediated p42 MAPK activation and HB-EGF mRNA induction. Genistein markedly inhibited both responses (Figures 7A and 7B), whereas cytochalasin B did not affect either kinase activation or mRNA induction (Figures 7C and 7D). In additional experiments, higher concentrations of cytochalasin B (up to 10 μM) were found not to inhibit wound-mediated p42 phosphorylation. The efficacy of the drug was demonstrated by its ability (at 1 μM) to completely prevent the restitution of individual wounds made in RIE-1 cell monolayers (results not shown).

DISCUSSION

Agonists that act through distinct receptor types induce rapid, generally transient, increases in cellular HB-EGF mRNA levels [5]. It is likely that the elevation in HB-EGF mRNA is due principally to increased gene transcription [20,36,37]. The intracellular signals by which various agonists activate the HB-EGF gene remain to be determined; several pathways have been implicated. There is evidence for an involvement of nuclear factor κB activation in the induction of HB-EGF mRNA in monocytes treated with platelet-activating factor [38]. The recent finding that transforming growth factor β induces HB-EGF gene expression in epithelial cells [39] suggests a possible role for the Sma-and-Mad-related-protein (Smad) pathway. The observations that HB-EGF mRNA levels are increased in cells transformed by the v-*ras* [15,40], v-*raf* [15] or v-*jun* [41] oncogenes have provided additional insight into the regulation of HB-EGF gene expression. In particular, the demonstration that HB-EGF is a Raf-1-regulated gene [15] points to the involvement of p42/p44 MAPKs in inducing HB-EGF gene expression.

It has been reported that p42/p44 MAPKs were rapidly activated in intestinal epithelial cell cultures in which multiple wounds had been made with a cell scraper [18] or razor blade [19]. Using a plastic comb as a convenient method to generate multiple wounds in an epithelial cell monolayer, we have extended these results to show that p42 MAPK activation by scrape-wounding is dependent on tyrosine kinase activity but is independent of PI-3K and the integrity of the actin cytoskeleton. The potential involvement of p42/p44 MAPK activation in HB-EGF gene induction [15,17] led us to hypothesize that HB-EGF mRNA might increase in wounded cell cultures. Using hybridization *in situ* and Northern blot analysis, we have established that HB-EGF mRNA levels increase rapidly in wounded cell monolayers. In addition, we found that two different MEK inhibitors, PD098059 and U0126, prevented HB-EGF mRNA induction in response to scrape-wounding. Because p42/p44 MAPKs are the only known substrates of MEK, our results suggest that the activation of this pathway is required for wound-mediated HB-EGF gene induction. However, an important caveat to this conclusion stems from the recent finding that activation of a novel member of the MAPK family, extracellular signal-regulated protein kinase 5 (ERK5), is potently inhibited by PD098059 or U0126 [42]. ERK5 is activated by various stress stimuli and by growth factors [42]. Additional studies will therefore be necessary to determine whether scrape wounding activates ERK5 and, if so, whether this kinase has a role in HB-EGF gene expression.

An interesting question concerns the nature of the upstream signals that link mechanical damage of cell monolayers with the rapid activation of the p42 MAPK. Experiments in which medium was transferred from wounded monolayers to unwounded cells have provided evidence both for [19] and against [18] the production of a soluble mediator by the wounded cultures. Immunostaining with a phospho-specific MAPK antibody demonstrated that the activation of p42/p44 was localized to cells at or near wound edges [18]. Consistent with this was our observation that HB-EGF mRNA levels increased in cells bordering the wounds. These results argue against the involvement of a widely diffusible factor. However, for technical reasons, these experiments were performed with single scrape wounds; it is feasible that additional pathways are activated in cultures that were subjected to multiple wounds to facilitate biochemical analyses.

The activation of p42/p44 MAPKs can occur through PKC-dependent or PKC-independent pathways. Down-regulating PKC by pretreating cells with PMA did not affect either p42 MAPK activation or the induction of HB-EGF mRNA in wounded monolayers. However, because not all PKC isoforms are down-regulated by this treatment, it is not possible to exclude the involvement of PKC solely on the basis of this result. We therefore attempted to test the role of PKC further by using Ro31-8220, which is a potent inhibitor of multiple PKC isoforms [29]. The drug did not inhibit p42 MAPK activation in wounded monolayers. Indeed, we observed a small increase in the conversion of p42 into pp42 MAPK in both control and wounded cultures that had been treated with Ro31-8220. We found that the activation of p42 MAPK by EGF was also increased by Ro31-8220 in RIE-1 cells. These results suggest that PKC is not required for the activation of p42 MAPK in response to monolayer wounding or treatment with EGF. In marked contrast with its effects on p42 MAPK activation, Ro31-8220 blocked the increase in HB-EGF mRNA induced by either wounding or EGF. This result could indicate that a PKC isoform that is inhibited by Ro31-8220, but is not down-regulated by PMA treatment, is required for HB-EGF mRNA induction, acting through a mechanism that is independent of p42 MAPK. Our results are reminiscent of findings reported by Beltman et al. [32], who found that, in Rat-1 cells, Ro31-8220 potentiated p44 MAPK activation by EGF and lysophosphatidic acid but strongly suppressed the expression of c-Fos and mitogen-activated protein kinase phosphatase 1 (MKP-1). However, neither PKC down-regulation nor the PKC inhibitor GF109203X affected c-Fos or MKP-1 expression, leading the authors to suggest that the effects of Ro31-8220 might be due to pharmacological activity unrelated to PKC inhibition. This proposal is supported by the recent findings that Ro31-8220 inhibits the activity of p70S6K, MAPK-activated protein kinase-1 (MAPKAP-K1) and mitogen- and stress-activated kinase-1 (MSK1) with similar potencies to PKC isoforms [30,31]. MAPKAP-K1 and MSK1 are both activated by p42/p44 MAPKs and might contribute to the induction of gene expression by this pathway [31,43]. Our finding that Ro31-8220 completely blocks HB-EGF mRNA induction, without inhibiting p42 MAPK activation, raises the possibility that MAPKAP-K1 or MSK1 might be required for HB-EGF mRNA induction.

p42/p44 MAPKs are activated by various mechanical stimuli in addition to scrape-wounding. For example, cyclic strain applied to smooth-muscle cells [44] or cardiac myocytes [45], fluid shear stress in vascular endothelial cells [35], pressure-loading of mesangial cells [46] and contraction of skeletal muscle *ex vivo* [47] all lead to rapid activation of the MEK/MAPK pathway. Genistein inhibited p42 MAPK activation by shear stress and pressure-loading, indicating a requirement for tyrosine kinase activity [35,46]. We found that genistein also inhibited wound-mediated p42 MAPK activation, although it remains to be established whether the same tyrosine kinase(s) are involved in the response to these stimuli. Activation of p42 MAPK by shear stress [35] or mechanical stretch [48] is inhibited when actin filaments are disrupted with cytochalasin. Our finding that p42 MAPK activation in response to scrape-wounding is unaffected by cytochalasin B suggests that distinct mechanisms underlie the activation of common signalling pathways by different mechanical stimuli.

Peptide growth factors have been implicated in epithelial regeneration after injury to skin [49] and to the mucosal surfaces of, for example, the gastrointestinal [50] and urinary [51] tracts. Platelets and inflammatory cells that accumulate at deep wound sites have been identified as an important source of these factors [49]. However, superficial epithelial damage must also be rapidly repaired to re-establish barrier function [50]. In the absence of vascular involvement, the complement of growth factors that is normally delivered to deeper wounds might not be readily available. In this situation, the local induction of growth factor expression within the damaged epithelium could be particularly important. During epithelial restitution, the elongation and migration of epithelial cells from the wound margins to cover the denuded area precedes compensatory cell proliferation [49,50]. Because HB-EGF potently activates cell migration [22,52] as well as cell proliferation, it is possible that the factor provides a dual stimulus for the key events of epithelial repair. It will be possible to use model systems [53] to test whether the ectopic over-expression of HB-EGF increases the rate of epithelial restitution, or whether blocking HB-EGF function with neutralizing antibodies delays the repair process.

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