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Injury and EGF Mediate the Expression of α6β4 Integrin Subunits in Corneal Epithelium

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Abstract Our goal was to evaluate the role of epidermal growth factor and injury on the expression of integrin subunits alpha6(α6) and beta4(β4). An in vitro wound model was used to evaluate corneal wound repair and cellular migration. Primary rabbit corneal epithelial cell cultures were serum-starved and injured in the presence or absence of EGF or tyrphostin AG1478, an inhibitor of EGF receptor kinase activity. Repair was monitored morphologically and expression was analyzed using in situ hybridization and immunohistochemistry accompanied by confocal microscopy. The addition of EGF to cell cultures induced a dose-dependent increase in beta4 mRNA expression but the constitutive expression of α6 was several fold greater. In the wounded cultures there was a rapid change in expression at the edge of the wound that was enhanced with EGF. In our model there was an increase in β4 and α6 protein in migrating cells. Changes in integrin expression were accompanied by a transient increase in activation of the EGF receptor. The addition of tyrphostin inhibited migration of cells and wound repair, the activation of the EGF receptor and phosphorylation of β4 in the cytoplasm. These data indicate that the activation of the EGF receptor plays a critical role in the regulation of integrin receptors and the mediation of cellular migration. J. Cell. Biochem. 80:397–414, 2001.

Key words: wound healing; α6β4; EGF; epithelium; migration

Cell migration is a fundamental process of wound healing in biological systems and is mediated by cytoskeletal elements [Lazarides and Revel, 1979; Kupfer et al., 1986], adhesion molecules, and the matrix that the cells migrate across. Regulation of cellular functions that occur in wound healing may involve both cell–matrix and cell–cell communication. Corneal wound healing commences when the stratified cells are disrupted and the cells migrate to fill the void. The intact cornea has 5–7 layers of stratified squamous cells of which the basal cells adhere to the basal lamina via hemidesmosomes. The integrin, (α6) and (β4), has been localized to hemidesmosomes and these molecules are involved in the assembly and disassembly of the adhesion complexes [Stepp et al., 1990; Jones et al., 1991]. The α6β4 integrin complex localized along the basal cell surface is altered with increasing age as shown using confocal microscopy and three-dimensional imaging [Trinkaus-Randall et al., 1993].

When a corneal abrasion occurs in vivo, and cellular migration commences, there is a detectable increase in vinculin in cells at the leading edge that is not detected either in unwounded epithelium or in an epithelium at a distance from the leading edge [Zieske et al., 1989]. In addition, Horiba and Fukuda [1994] have shown that vinculin and α5β1 were localized on the basal and lateral surfaces of regenerating epithelial cells in rat tracheal cells. In contrast, bullous pemphigoid antigen, is present in intact epithelium and becomes redistributed under migrating cells [Gipson et al., 1993]. Numerous studies have examined the changes in levels of integrin proteins in response to wounding [Grushkin-Lerner and Trinkaus-Randall, 1991; Horiba and Fukuda 1994; Stepp

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et al., 1996]. The results of these studies have depended on the model chosen to evaluate the response. Grushkin-Lerner and Trinkaus-Randall [1991] used an in vivo model and showed that the localization of specific integrin subunits changed with time after the injury and with the extent of the injury and this was confirmed by Stepp et al. [1996]. Growth factors and cytokines have been shown to modulate a number of integrin-dependent functions including cell adhesion, cell migration, and cytoskeletal organization [Chen et al., 1993; Mathay et al., 1993; Klemke et al., 1994; Kinashi et al., 1995; Serve et al., 1995; Lafrenie and Yamada, 1998; Aplin and Juliano, 1999].

Epidermal growth factor (EGF) is a single chain polypeptide known to play a role in proliferation and differentiation of epithelial cells in many tissues [Savage and Cohen, 1973; Ho et al., 1974; Catterton et al., 1979; Das, 1982; Barrandon and Green, 1987; Carpenter, 1987; Watanabe et al., 1987; Hebda, 1988]. Both EGF and TGF-α have been shown to enhance re-epithelialization in corneal wounds [Schultz et al., 1991]. In vivo experiments have been conducted where EGF was added to determine its efficacy in enhancing wound repair and strength [Leibowitz et al., 1990]. Recently, studies have demonstrated that the tyrosine kinase activity of the EGF receptor is critical for EGF signal transduction. Upon activation by a ligand, the kinase is activated and EGFR tyrosyl phosphorylates itself along with other effector molecules [Ezeh and Farbman, 1998; Wilson and Gibson, 1999].

We have developed an in vitro wound model to evaluate the relationship between injury and the expression of α6β4. Specifically, we evaluated both the role of EGF on integrin expression and activation, and the role of injury on the activation of the EGF receptor. When cells were wounded in serum-free medium we found that the injury alone altered the localization of α6 and β4 mRNA rendering it present along the wound margins. Injury itself increased the availability of binding sites and induced the activation of the EGF receptor. The presence of exogenous EGF enhanced the expression of integrin subunits and receptor activation. Cells migrated preferentially to EGF and those that migrated showed a change in the localization of the protein, α6 and β4. These findings have implications in the regulation of wound repair.

METHODS

Cell Culture and Wound Model

New Zealand rabbit eyes were obtained from Pel Freeze (Rogers, AK). The eyes were shipped on ice and used within 24 h of enucleation. Epithelial cell cultures were established from explants according to Trinkaus-Randall et al. [1990]. Briefly, the epithelium was removed, and cultured in Dulbecco's Modified Eagle's Medium containing 4% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, 0.25% antifungal agents, and 1% non-essential amino acids (GIBCO, Grand Island, NY).

To assess the cellular response to injury we developed an in vitro wound model [Haq and Trinkaus-Randall, 1998; Song et al., 2000]. Cells were subcultured and at confluence were serum-starved for 24 h. The conditioned medium was removed prior to injury, replaced with phosphate-buffered-saline pH 7.2 (PBS), and linear wounds were made. When the injury response was assessed by northern blot analysis, the cultures were wounded every 2 mm with a 25G needle to create an injured state [Song et al., 2000]. When cells were assessed morphologically a single injury was made. The conditioned medium was added back to the cells and the cells were evaluated at several time points over 24 h. To determine if the response to epidermal growth factor (EGF) was dose-dependent, experiments were conducted in a conditioned medium containing concentrations ranging from 0 to 100 ng/ml.

Probes

The plasmid α6.1 pTZB was a gift from Dr. Tamura. A 300 bp α6 cDNA restriction fragment (SmaI) was generated for the nonisotopic in situ hybridization experiments. A 1.2 kb restriction fragment (XbaI) was generated and used for northern blot analysis. The plasmid pGEM1-b4ES1 was a gift from Dr. Suzuki. A 600 bp cDNA restriction fragment (SacI/SmaI) was generated and used for both in situ hybridization and northern blot analysis. A 488 bp cDNA of 18S rRNA was generated by RT-PCR. The sequence of the upstream 18S rRNA primer was 5′-TCA AGA ACG AAA GTC GGA GG-3′ and the sequence of the downstream 18S rRNA primer was 5′-GGA CAT CTA AGG GCA TCA CA-3′.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated from the wounded cultures of epithelial cells using TRIzol Reagent (GIBCO/BRL). Fifteen micrograms of total RNA was denatured and separated by electrophoresis using a 1% agarose gel containing 1.9% formaldehyde. Equal gel loading and integrity of 18S and 28S RNA was verified using ethidium bromide. RNA was transferred to a Duralose membrane (Stratagene, La Jolla, CA) and UV cross-linked. Blots were hybridized with α6 and β4 cDNA probes or with 18S rRNA probes using the Radprime DNA labeling kit (Pharmacia, Piscataway, NJ). Prehybridization and hybridization at 68°C were performed in Rapid-Hybridization buffer (Stratagene, La Jolla, CA). The blots were washed twice at room temperature in $2 \times$ SSC buffer ($1 \times$ SSC: 0.15 M NaCl, 15 mM sodium citrate) containing 0.1% sodium dodecyl sulfate, once in $1 \times$ SSC buffer containing 0.1% sodium dodecyl sulfate at 68°C, and once in $0.1 \times$ SSC containing 0.1% sodium dodecyl sulfate at 60°C. Membranes were exposed to Amersham Hyperfilm for 24–48 h and hybridizing bands were quantified using NIH Software. Values for integrin subunits, β4 or α6, were normalized to values for the 18S rRNA. Statistical analysis was performed using a Student's *t*-test to compare wounded and unwounded groups. Differences were considered significant when $P < 0.05$.

Nonisotopic In Situ Hybridization

Replicate experiments were performed to evaluate the localization of cells expressing α6 and β4 in response to injury. Non-isotopic in situ hybridization for α6 and β4 mRNA was conducted [Singer et al., 1986; Grushkin-Lerner et al., 1997]. Briefly, cells were fixed in 4% paraformaldehyde containing 5.0 mM $MgCl_2$ pH 7.4 for 15 min., washed, and stored in PBS at 4°C overnight. Prior to hybridization, cells were incubated in Tris–HCl, pH 7.4, 0.1M glycine. The cDNA restriction fragment of α6 and β4 was prepared and labeled using the Nick Translational Kit (Boehringer Mannheim, IN) and Digoxigenin-11-UTP. Southern blot analysis of the labeled probe was conducted to ensure that the probe was labeled specifically. The cDNA probe melted in 100% formamide at 90°C was combined with an equivalent volume of hybridization buffer containing $20 \times$ SSC, 2% BSA, 50% dextran sulfate, and VRC (vanadyl ribonucleoside complex) at a ratio of 1:1:2:1 and added to cells and incubated in a humidified chamber at 37°C for 15 min. After hybridization and extensive washing with $2 \times$ SSC, the cells were incubated in anti-digoxigenin-FITC for 1 h at 37°C.

Cells were washed, cover-slipped, and images were recorded using a Zeiss LSM 510 (2.5) Confocal Laser Scanning Microscope. For these experiments a single image was taken at the level of the nucleus at the same depth from the apical surface for each image. The pixel intensities of cells at the leading edge and one cell back were measured. Each image contained a variable number of cells ranging from 2 to 25. The intensity of fluorescence was measured using the LSM 510 software where the mean pixel intensity per square micrometer (mpi/a^2) was determined for each of three images and an average of these three measures was recorded. Control cells (those without cDNA) were analyzed first and the gain was set for the experimental images where the fluorescence of the control was negligible. For the wounded images, the wounded region was determined and subtracted from the total area. All images were printed under the same conditions. Simultaneously transmitted light images were recorded to calculate the total number of cells in each field. A minimum of 50 cells was counted for each parameter.

Immunohistochemistry

Cells were cultured in chamber slides for 24 h serum-free medium. The procedure for staining corneal epithelial cell populations was described previously [Trinkaus-Randall et al., 1991, 1993]. Briefly, cells were fixed with 4% paraformaldehyde in PBS pH 7.4 (freshly made) at room temperature for 15 min and rinsed three times with PBS containing 3% bovine serum albumin (BSA). The cells were incubated in 1% BSA containing monoclonal antibodies (MAb) to α6 (Chemicon), β4 (Transduction Labs) or EGF receptor (Transduction Labs) overnight at 4°C, rinsed with PBS and washed for 10 min in 3% BSA/PBS. Cells were then incubated with either FITC goat anti-rat IgG (1:100) or FITC horse anti-mouse IgG (1:100) for 1 h on a rocker at room temperature. Double staining experiments were also conducted and in these experiments the lasers were set so that cross-over was not detected in either channel. To perform

these experiments, cells were stained with a MAb directed against β4 and then the respective secondary antibody conjugated to FITC and secondly with a polyclonal antibody to phosphorylated tyrosine residues [ptyr] (Transduction Labs) and then the respective secondary antibody conjugated to Cy3 (1:200). All secondary antibodies were purchased from Jackson Labs. Controls were incubated with either pure rat IgG or pure mouse IgG. All experiments were repeated a minimum of three times.

Morphological Assessment of Wound Assay

To analyze the migration of cells in response to injury, images were taken with a SPOT Digital Camera system that is linked to a Nikon Diaphot Inverted Microscope equipped with epifluorescence. Wounds were created as described previously and the same region was followed over time. Quantification of cell migration was performed with a 1 mm × 1 mm grid that was placed over the injury area. To locate the same injury area, two 1–2 cm long fine scratches were made to form a cross line with a diamond pencil. Each value represents the mean of three different experiments with a minimum of 500 cells counted per experiment.

Migration Assay

Cells were serum-starved for 24 h, harvested, and seeded into Transwell migration chambers. Trypsin was inhibited with a soybean trypsin inhibitor. Cells migrated to positive control (10% serum), negative control (0% serum) and to four concentrations of EGF (0.1,1,10 and 100 ng/ml) diluted in binding buffer (2 mM gelatin, 0.1 mM HEPES in DMEM) for 5 or 12 h. Non-migrated cells were scraped off filters in 3.7% formaldehyde pH 7.2 for 15 min at room temperature. Migratory cells were stained with propidium iodide (Molecular Probes, Inc., Eugene, OR) for 10 min at room temperature and mounted on slides. Images were taken with a Spot digital camera system. The total number of migratory cells was counted. In additional experiments, migratory and non-migratory cells were double-stained with MAb to integrin subunits α6 or β4 and images were taken using a Spot camera. Image analysis was performed and fluorescence intensity was determined at a defined perinuclear region and at regions along the plasma membrane. The ratio of plasma membrane: perinuclear (pixel intensity) was determined to calculate changes in localization. The computer-generated programs give the value as pixel intensity units. Background levels of pixel intensity outside the cell also were taken and subtracted from the pixel intensity of the specific region of the cell.

Localization of EGF Receptors

To localize EGF binding sites at various times after injury, wounded and unwounded cultures were incubated in DMEM and EGF (25 nM) at 4°C for 2.5 h in binding buffer (DMEM, 0.05% gelatin and 25 mM HEPES) [Song et al., 2000]. Cells were rinsed twice with binding buffer and PBS and fixed for 10 min with formaldehyde (3.7% in PBS pH7.2). Cells were rinsed again in PBS and blocked with PBS-BSA (3%) for 1 h at room temperature. Anti-EGF antibody (150 μg/ml) in PBS–BSA (2%) was hybridized for 1 h at 37°C. After three washes with PBS–BSA (2%) and two washes with PBS, the secondary anti-IgG-FITC (1:100) in PBS–BSA (2%) was hybridized for 1 h at 37°C. Cells were washed and anti-fade (Molecular Probes Eugene, OR) was added to the coverslip.

Confocal Microscopy

Cells were imaged on a Zeiss inverted LSM 510 confocal laser scanning microscope (CLSM) equipped with an Argon laser and 2 He lasers. All experimental cultures were examined under the same conditions [Trinkaus-Randall et al., 1993; Wu et al., 1995; Grushkin-Lerner et al., 1997, Song et al., 2000]. Experimental cultures or slides were normalized to control cultures as the control cultures (lacking either primary antibody or cDNA probe) were set so that fluorescence was negligible. All images were acquired using an optical slice of 1.5 microns. Image analysis was performed using Zeiss LSM510 software 2.5 where the mean pixel intensity per unit area (mpi/a^2) was measured for each field. The background staining for the secondary antibody was set at 0 mpi/a^2. All experimental images were then captured at these control settings. Images were stored on zip or jazz disks and transferred to a Macintosh workstation, where images were configured in Adobe Photoshop.

RESULTS

Our goal was to evaluate the changes in the expression of α6 and β4 integrin subunits in response to injury and/or EGF and to determine if injury itself altered the occupancy of EGF receptors. Experiments were conducted in the absence of serum to evaluate if soluble wound mediators played a role in regulating integrin subunit expression. Previously, α6 and β4 integrin mRNA were shown to be expressed constitutively in cultured epithelial cells [Grushkin-Lerner et al., 1997].

Change in β4 and α6 mRNA is Mediated by EGF and Injury

To evaluate if the expression of α6 and β4 was mediated by EGF, wounded and parallel unwounded cultures were incubated for 3 h in the presence or absence of EGF (0, 5, 10, 15, 25, 50 and 100 ng/ml) and evaluated using northern blot analyses. Response in these wounded cultures represented an average of the entire culture including wounded and unwounded cells. The integrity of RNA was monitored using ethidium bromide staining of 18S and 28S and expression was normalized to 18S rRNA. At 3 h there was no significant change in β4 mRNA in response to injury alone (Fig. 1A, B). EGF caused a dose-dependent response in β4 mRNA. When cells were incubated in 25 ng/ml EGF, there was a two-fold increase in the expression of β4 mRNA in wounded cultures compared to unwounded cultures suggesting a synergistic relationship between EGF and injury. At higher concentrations of EGF there was a four-fold increase in β4 mRNA in wounded cultures but there was also a large increase in the expression of unwounded cells. When the expression of α6 was evaluated in response to EGF, there was a negligible increase (1.3) in expression of the unwounded epithelial cultures in response to EGF; however, the level of constitutive α6 (-EGF) was significantly greater than the level of β4 (Fig. 1C, D). The wounded population of cells exhibited maximal α6 expression in a medium containing 50 ng/ml EGF (two-fold increase). Therefore the same concentration of EGF did not result in maximal expression of both α6 and β4 integrin. These results indicate that α6 is expressed constitutively.

To follow changes in localization of the integrin subunits over time, the cultures were incubated as described previously in the presence or absence of EGF and evaluated using in situ hybridization. Experimental cultures were normalized to controls lacking a cDNA probe. Unwounded populations of cells displayed low levels of expression of β4 mRNA that did not change over a period of 24 h (Fig. 2). There was a rapid change in expression 5 min after injury (in the absence of EGF), as only a single row of cells at the leading edge of the injury expressed β4 mRNA (arrows) (Fig. 2). When EGF (25 ng/ml) was added to wounded cultures, the percentage of cells expressing mRNA increased from 39 to 55% of the cells but remained localized to the wound edge (arrows). As time after injury increased from 5 to 30 min, the percentage of cells in the field expressing β4 mRNA increased from 39 to 63% in the absence of EGF and from 55 to 76% in the presence of EGF (arrows). In addition, there was a 1.3- and 1.7-fold increase in mpi/a^2 in wounded cultures (absence and presence of EGF, respectively), while there was no change in the unwounded cultures. At 1 h there were more than three rows of cells distal to the leading edge that expressed β4 mRNA in the absence of EGF that increased with a growth factor. By 3 h, more than 10 rows of cells distal to the leading edge expressed β4 mRNA. The increase was transient and at 6 h there was only minimal difference between experimental and control cultures (data not shown). By 24 h, fluorescence was negligible in the wounded cultures but returned to control levels by 48 h (data not shown).

While in situ analysis provides information regarding the position of the expressing cells with respect to injury and time, northern blot analysis was also conducted on the entire cell population to evaluate the effects of injury and EGF on the overall β4 mRNA levels. Densitometric analysis was performed and changes in β4 were normalized to 18S rRNA. Maximal expression of β4 mRNA was detected 6 h after injury when exogenous EGF (25 ng/ml) was present. Since the northern blot analyses represent an average of the entire culture that includes both wounded and unwounded cells, we hypothesize that the time course in the northern blot is delayed compared to in situ hybridization because the increase in the expression induced by the injury is masked by the unwounded cells in the culture. When exogenous EGF was added and cells were cultured, there was a three-fold change in expres-

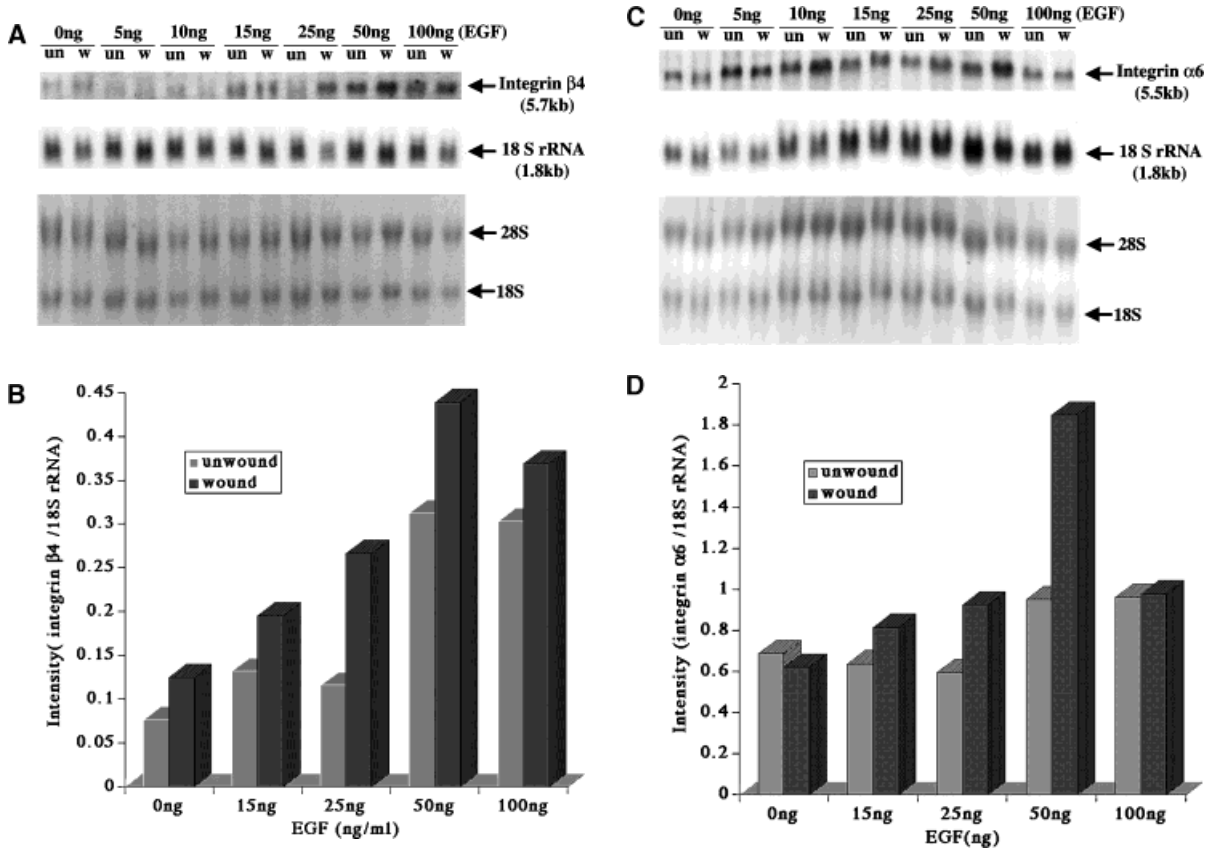

Fig. 1. EGF mediates integrin expression. Northern blots were probed with β4, α6 and 18S rRNA cDNA probes. Multiple wounds were made throughout the culture and cells were cultured for 3 h in the presence or absence of EGF (0–100 ng/ml). RNA extractions represent the entire culture. Northern blots and respective densitometric analyses were performed where expression of integrin subunit was normalized to 18S rRNA. Ethidium bromide staining of 18 and 28S indicate integrity of RNA. (**A** and **B**) β4 mRNA expression (normalized to 18S rRNA) was elevated in response to injury and EGF and the maximal difference between wounded and unwounded was detected at 25 ng/ml EGF. (**C** and **D**) α6 mRNA expression (normalized to 18S rRNA) displayed enhancement in the wounded cultures at higher concentrations of EGF. Data are representative of three experiments.

sion at 3 and 6 h (Figs. 2 and 3A). At 6 h both the wounded and unwounded cultures containing EGF demonstrated enhanced expression. However, expression by the wounded (+EGF) cultures was more than two-fold greater than wounded cultures lacking EGF and more than four-fold greater than unwounded cultures lacking EGF (Fig. 3A). The integrity of RNA was monitored using ethidium bromide staining of 28S and 18S. In addition there was no significant change in 18S rRNA over time or condition. These results indicate that the change in expression that is altered with the injury itself is small but there is a synergistic response with exogenous EGF.

When the localization of α6 mRNA was evaluated, there were negligible changes in either the percentage of cells expressing α6 mRNA or in the mpi/a^2 in the unwounded population. A similar trend that was seen with β4 was detected here where there was a distinct response to injury that was manifested with an increasing number of cells expressing α6 mRNA from 5 min to 3 h. Immediately after injury 24 and 26% of the cells expressed α6 mRNA at the leading edge (absence or presence of EGF, respectively) (arrows). By 30 min there were two to three rows of cells in wounded cultures or a 2.6-fold increase in cells expressing α6 mRNA (arrows) (Fig. 4). By 24 h, the response was similar to the phenomenon observed with β4 (Fig. 4). Northern blot analysis was conducted on the entire culture to evaluate the average effect of injury and EGF on the expression of α6 mRNA. Densitometric analysis was performed and the change in α6 was normal-

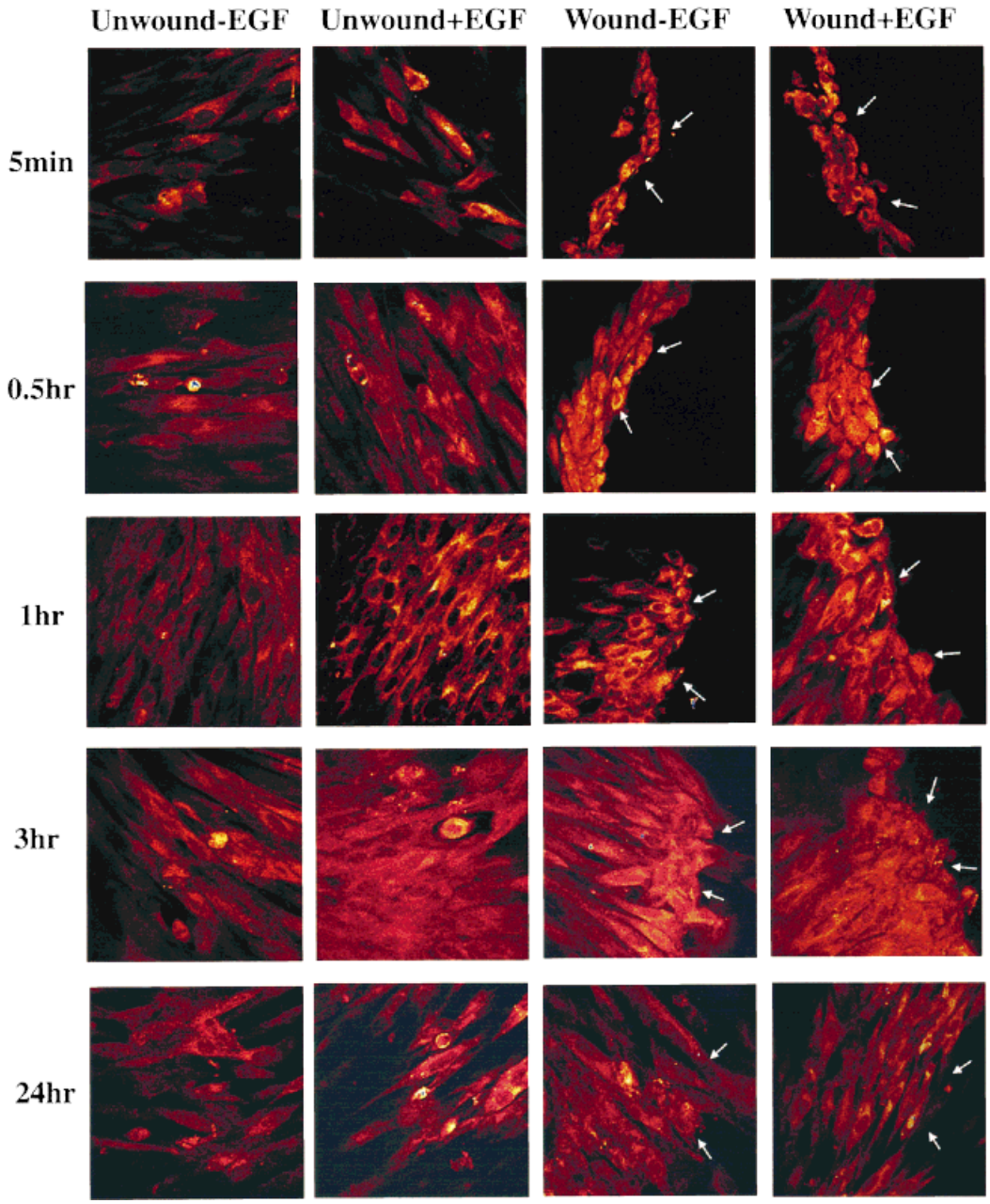

Fig. 2. Localization of β4 mRNA in wounded and control cultures using non-isotopic in situ hybridization and imaged with confocal microscopy. Confocal images represent single images taken the same depth from the apical surface. Cultures were serum-starved, injured, and incubated in the presence and absence of EGF (25 ng/ml) over 24 h. The leading edge of the wound is demarcated by arrows. β4 was expressed at the leading edge of the wound by 5 min and became intense by 30 min with an associated decrease a distance from the wound edge. Expression was enhanced when exogenous EGF was present. Expression was detected in cells distal to the wound edge at 1, 3 and 24 h. β4 mRNA was present at background levels in unwounded cultures. (×82). The images are representative of three individual experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ized to 18S rRNA. The results indicate that α6 is expressed constitutively at relatively higher levels than β4 mRNA over time after injury but the relative increase in expression of α6 mRNA is dependent on exogenous EGF (Fig. 3B).

α6 and β4 Protein in Response to EGF and Migration

To evaluate the role of EGF on the expression of α6 and β4 protein, migration experiments

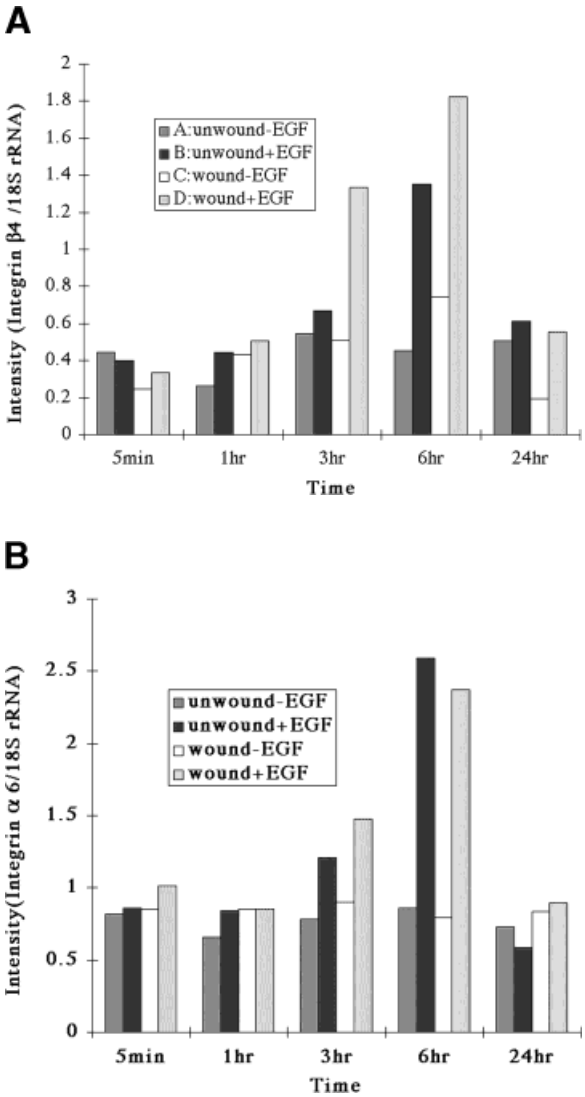

Fig. 3. Changes in integrin β4 and α6 mRNA expression over time in response to injury in the presence or absence of EGF (25 ng/ml). Multiple wounds were made throughout the culture and cells were cultured for 24 h in the presence or absence of EGF. RNA extractions represent the entire culture and northern blots and respective densitometric analyses were performed where expression of integrin subunit was normalized to 18S rRNA. Ethidium bromide staining of 18 and 28S indicate integrity of RNA. **A:** Densitometric analyses (normalized to 18 S rRNA) demonstrate that expression of β4 mRNA is enhanced 3–6 h after injury or during times of active migration. Expression was greatest when the injured cells were cultured in the presence of EGF and by 6 h, control unwounded cultures also expressed β4 at enhanced levels. **B:** Densitometric analyses (normalized to 18 S rRNA) showed enhanced expression (greater than unwounded control) at 3 and 6 h.

were performed and cells were stained with antibodies directed against α6 or β4. Previously, investigators in our laboratory and others have evaluated the localization of α6 and β4 in response to injury in vivo and in vitro [Grushkin-Lerner and Trinkaus-Randall, 1991; Stepp et al., 1996; Horiba and Fukuda 1994]. Our goal was to examine if migration altered the localization of β4 and if the integrin subunits were upregulated in response to EGF. The migration experiments were performed because in the injury experiments it was difficult to distinguish expression between the cells that were migratory and those that were simply adjacent to migratory cells. Consequently, a migration assay was developed where cells were migrated to EGF (see below). Migration to EGF and the expression and localization of α6 and β4 were evaluated in migratory and non-migratory cells.

Migration assays were performed to distinguish if cells migrated preferentially to EGF and to determine the expression of the migratory cells. Primary corneal epithelial cells were serum-starved for 12 h and cells were placed into the top of the migration chamber while EGF was added to the bottom. Cell migration was shown to be optimal (defined as number of cells that migrate to positive control vs. negative control) when assays were conducted for 12 h using 8 μm pore size membranes. Migration was defined by staining the membranes with propidium iodide and counting nuclei on 10 randomly chosen fields using a Spot Camera system. Migration was maximal at 10 ng/ml EGF and cells migrated to serum (positive control) with minimal migration to binding buffer (Fig. 5A). In addition, cells did not migrate to platelet-derived growth factor (PDGF) at a similar range of concentrations (data not shown) or to tyrphostin AG 1478 (1 μM) (Calbiochem) (data not shown).When the migratory and non-migratory cells were fixed and stained with antibodies directed against α6 or β4 we found that there was a change in the localization of the protein when they transitioned from a non-migratory to a migratory state (Fig. 5B). To normalize staining, the fluorescence of the non-migrated cells was used as the baseline and migrated cells were evaluated at the same settings. Migrated cells showed extremely high fluorescence compared to non-migratory cells. Change in localization was quantitated by taking the pixel intensity of a rectangle at the plasma membrane (A) and along the perinuclear/cytoplasm margin (B) of 50 cells and calculated the ratio of the mean pixel intensity of A:B (arbitrary units). Note that the data repre-

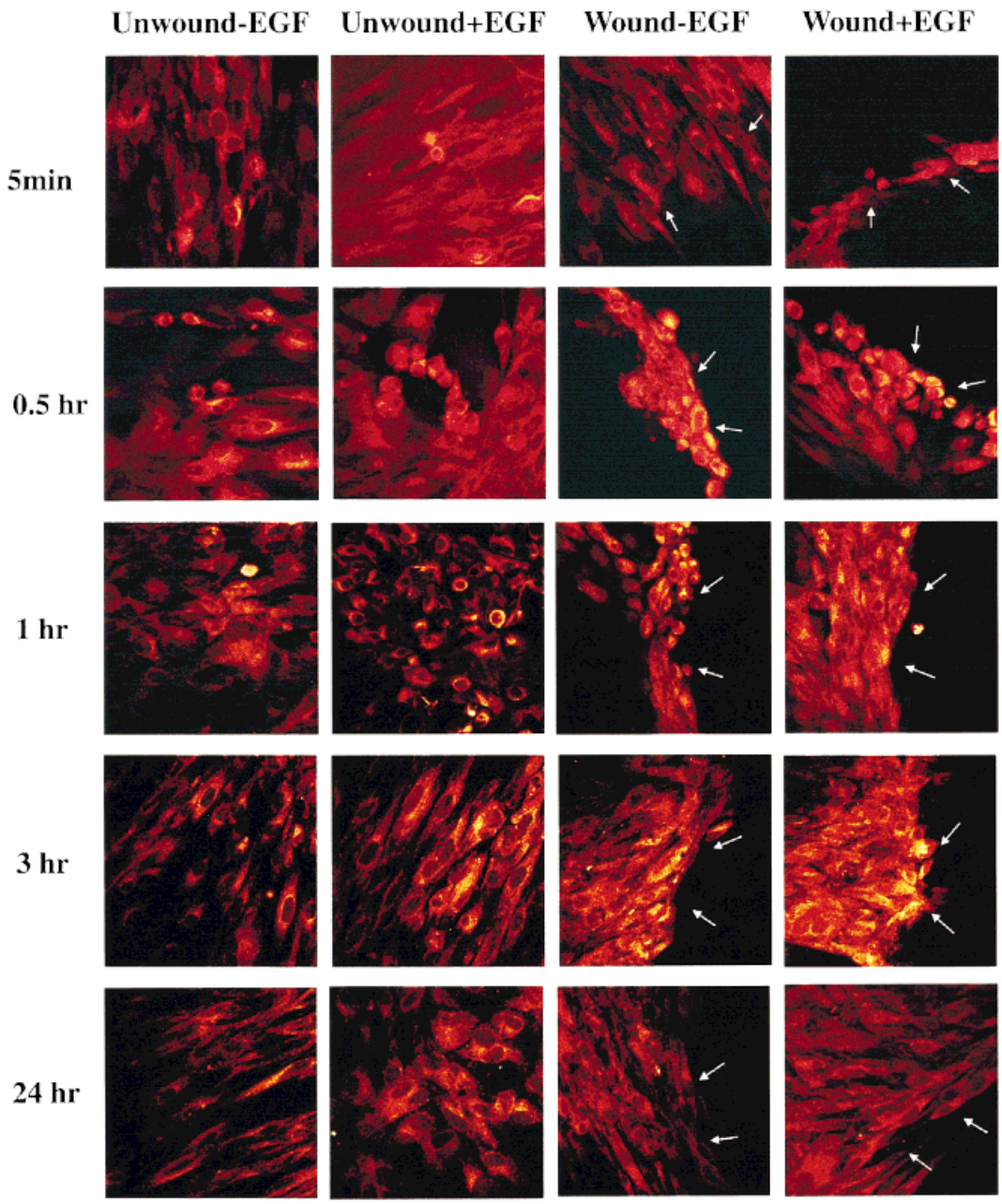

Fig. 4. Localization of α6 mRNA in wounded and control cultures using non-isotopic in situ hybridization and imaged with confocal microscopy. Confocal images represent single images taken the same depth from the apical surface. Cultures were serum-starved, injured, and incubated in the presence and absence of EGF (25 ng/ml) over 24 h. The leading edge of the wound is demarcated by arrows. Control cultures were not wounded and are represented at the same time points. Changes in α6 mRNA are not detected in the unwounded cultures over time and are slightly elevated at 3 h. There is a change at the leading edge when expression of distal cells was absent. This was enhanced when exogenous EGF was present (× 82). The images are representative of three individual experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

sents the percentage of cells that were migratory or non-migratory for each condition. Non-migratory cells had a ratio of 0.5:0.6 for both α6 or β4 indicating diffuse staining with the response independent of EGF concentration. When the cells migrated, the staining pattern of α6 and β4 was altered with intense staining along the plasma membrane compared to the perinuclear region and the value of the ratio increased (Fig. 5B). For β4, the increase in the ratio correlated with migration alone, while for α6 the increase in the ratio was only

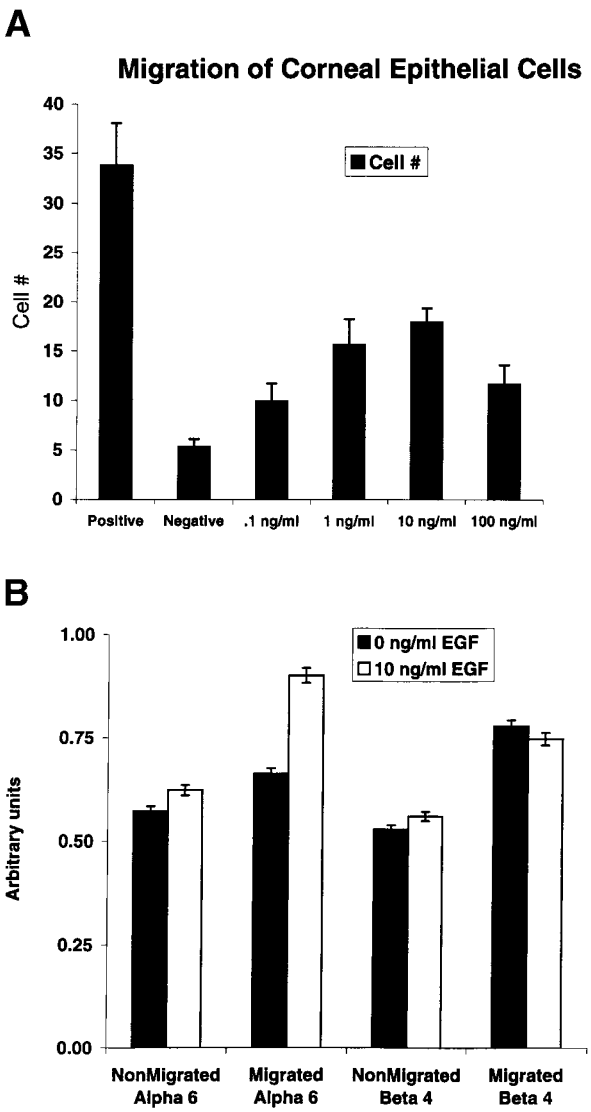

Fig. 5. Migration of cells to EGF and changes in the localization of α6 and β4 integrins in migratory and non-migratory cells. **A:** Cells were serum-starved, added to Costar polycarbonate membranes, and EGF was added to the bottom well at varying concentrations. Cells were also migrated to 10% serum or binding buffer. The membranes were cut out after completion and the non-migratory cells removed, cells fixed, stained with propidium iodide and nuclei counted. The data represent the average of 10 frames ± SD. **B:** Parallel experiments were conducted in duplicate and the fixed membranes were stained with MAbs directed against β4 and α6. Both migratory and non-migratory cells were analyzed and fluorescence was standardized by using the settings established for the non-migratory cells. Distinct regions along the plasma membrane or cytoplasm were evaluated for fluorescence intensity and the ratio of plasma membrane:cytoplasmic staining determined. The data represent the average of 10 frames ± SD and are representative of three independently conducted experiments.

detected when cells migrated to EGF. These results support the in vitro wounding assays where heterodimer was enhanced when cells were injured and allowed to heal in the presence of EGF (data not shown). These results indicate that expression is mediated by components of both active cellular migration and exposure to EGF.

Receptor Localization, Activation and Binding

Experiments were performed to determine whether local injury induced changes in the activation of EGF receptors and/or in the binding of exogenous EGF after injury. Activation was assessed using an antibody directed against the activated EGF receptor. The relative intensity of EGFR phosphorylation was evaluated using Zeiss LSM 510 software to calculate the mpi/a^2. In wounded cultures the value of a rectangular area was determined at the wound edge and the rectangle was then moved to a region back from the wound and the value determined for each time point and condition. This was compared to similar areas taken of unwounded cultures. When wounded (arrows) cultures were incubated in serum-free/EGF-free medium, 62% of the total number of cells displayed fluorescence 30 min after injury and neither the percentage of cells nor the mpi/a^2 changed after 1 h (Fig. 6) There was no change in the unwounded cultures in the same medium over time (data not shown). When wounded cultures were incubated in the presence of EGF there was a 2.5-fold increase in fluorescence intensity over control injury indicating enhanced phosphorylation of receptor (Fig. 6). Fluorescence intensity diminished to 50% of the control when cells were incubated in medium containing 1 μM tyrphostin AG1478 prior to injury (arrows at wound edge). When cells were inhibited in medium containing EGF, the mpi/a^2 was similar to control wounded cultures, but the number of cells expressing activated EGFR decreased by 25% (Fig. 6). Additional experiments were conducted to determine whether activation of the EGF receptor was necessary for cell migration. In these experiments cells were pre-incubated in 1 μM tyrphostin and cell migration was compared after injury in the presence or absence of EGF (Fig. 7). Cells incubated in tyrphostin did not heal while those incubated in EGF had an enhanced rate of repair (Fig. 7). Together these results indicate that the EGF receptors are activated with

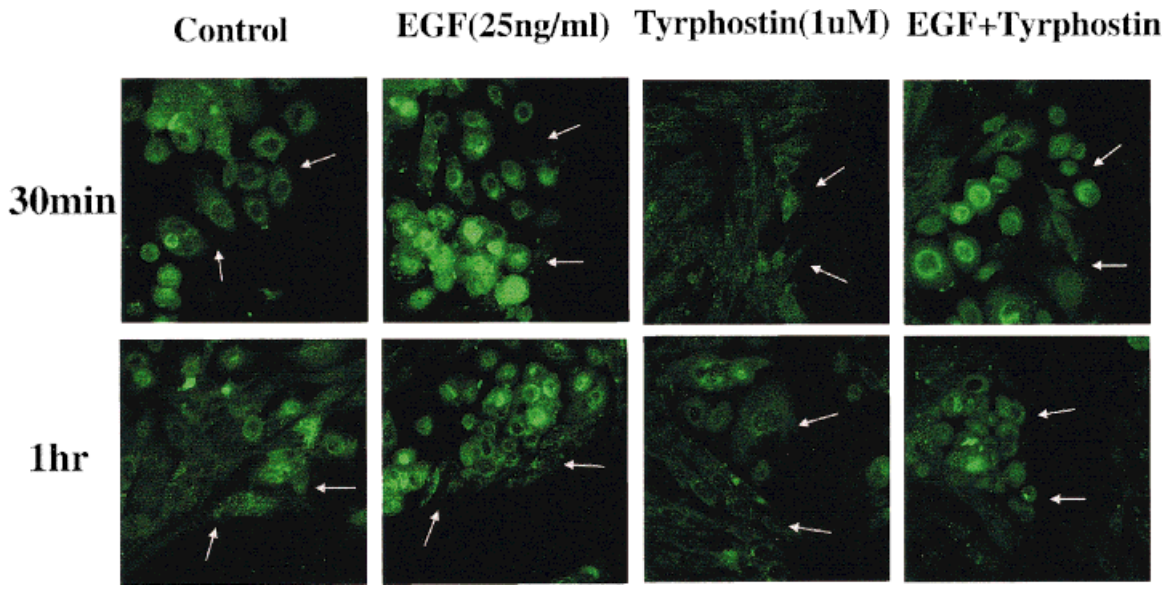

Fig. 6. Activation of the EGF receptor in response to injury. Confluent primary epithelial cells were serum-starved, injured, and incubated in the presence or absence of EGF and/or tyrphostin. EGFR expression was evaluated using an antibody directed against the activated EGF receptor and imaged using confocal microscopy. Confocal images represent the composite of z-series images. The leading edge of the wound is demarcated by arrows. The activation of the EGFR was enhanced with EGF and inhibited with tyrphostin (× 82). The images are representative of three individual experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

injury and play an active role in cellular migration and wound repair.

To visualize EGF bound to the cell surface and to prevent receptor internalization and growth factor processing, the localization of EGF bound was analyzed at 4°C. Binding experiments have been performed previously using immunohistochemical technology [Song et al., 2000]. Binding was detected in both the EGF-treated and wounded cultures at the leading edge (arrows) within 15 min after injury and was maximal at 30 min increasing by 35% (arrows) (Fig. 8). Fluorescence intensity decreased with distance from the wound and with increasing time after injury. By 6 h, fluorescence was similar in both wounded and unwounded cultures. The addition of exogenous EGF did not alter the overall response but did increase the number of fluorescent cells by 10%. These data indicate that the injury transiently altered the availability of receptors. By 6 h, fluorescence was negligible under all conditions. Fluorescence for the experiment was normalized to the secondary antibody controls where fluorescence was set to negligible levels. Fluorescence was not detected when EGF was omitted in the binding buffer (data not shown).

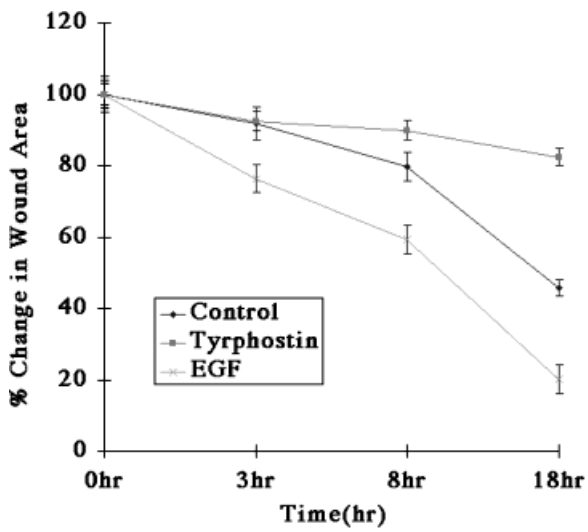

Fig. 7. Cellular migration that ensues after injury is mediated by the EGFR. Confluent primary epithelial cells were serum-starved, injured, and incubated in the presence or absence of EGF or tyrphostin. Micrographs were taken of the same field of cells at 0, 3, 8 and 18 h after injury. The graph represents the change in wounded area over time. Wound repair was enhanced with EGF and inhibited with tyrphostin compared to control wounded cultures.

To evaluate if the expression and activation of β4 was mediated by the EGF receptor, injury experiments were conducted in the presence or absence of EGF and/or tyrphostin. When cultures were evaluated 3 h after injury in serum-free medium alone, β4 and phosphorylated tyrosine residues were detected and the merged images showed corresponding co-localization in

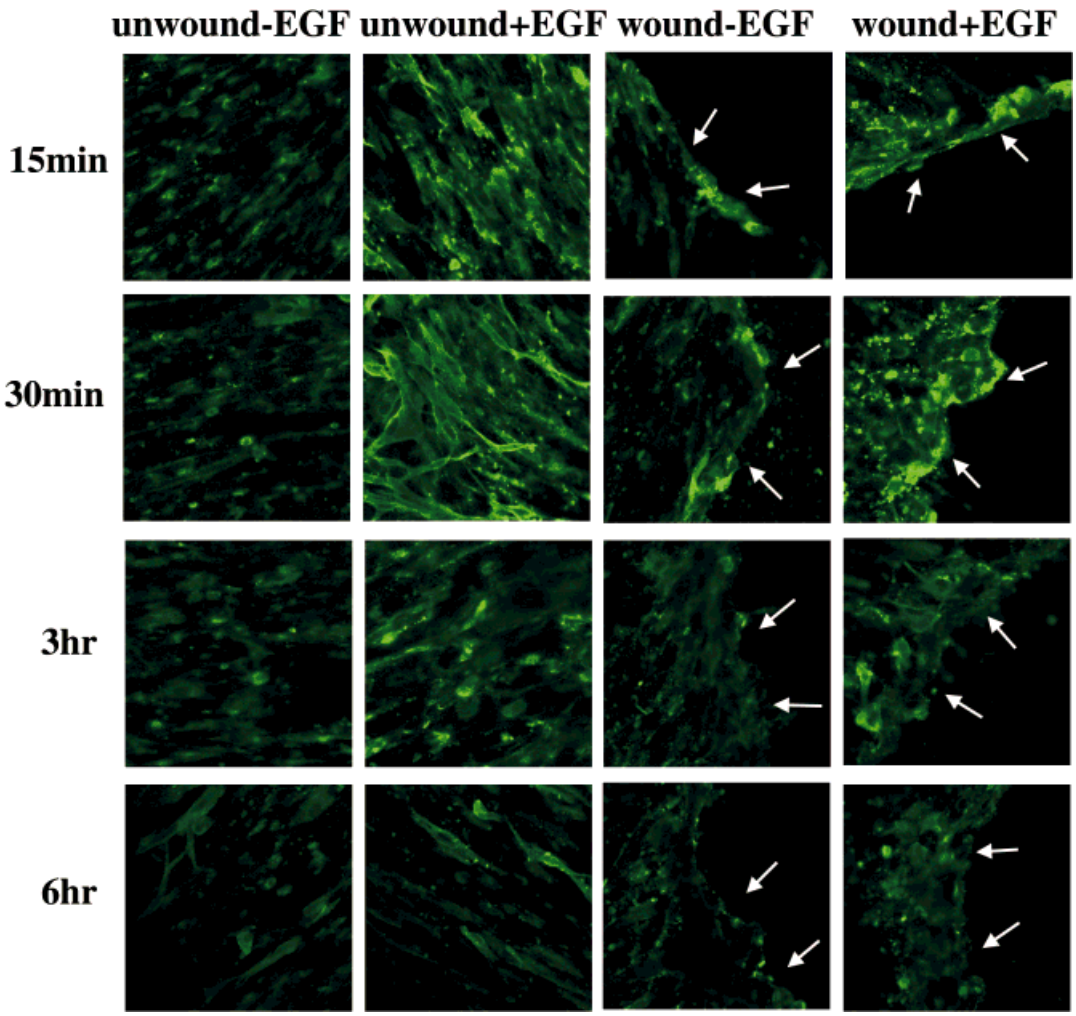

Fig. 8. Local injury-induced changes in binding of EGF to epithelial cells. Serum-starved epithelial cells were wounded and incubated for the times indicated (15 min to 6 h). Cultures were then incubated for 2.5 h at 4°C in presence of EGF. Cells were fixed and images were evaluated using confocal laser scanning microscopy. Confocal images represent the composite of z-series images. The leading edge of the wound is demarcated by arrows. Enhanced staining is detected at 15 and 30 min after injury (wound–EGF) indicating a change in occupancy of receptors (× 82). Experiments are representative of three independent experiments.

the cytoplasm indicating phosphorylation of β4 (Fig. 9). The presence of exogenous EGF enhanced the expression of β4, phosphorylation of tyrosine residues and phosphorylation of β4 (Fig. 9). When the phosphorylation of the EGF receptor was inhibited by tyrphostin, phosphorylation of tyrosine residues was negligible and the presence of β4 resembled injury control (Fig. 9). When the control experiment was performed where cells were preincubated in tyrphostin and injured in medium containing EGF the response was again inhibited (data not shown). These data indicate that while β4 and tyrosine phosphorylated residues are detected in response to injury, and that β4 is phosphorylated, they are upregulated by EGF and mediated by the EGF receptor.

DISCUSSION

In this study we evaluated the role of EGF on the expression of integrin subunits, β4 and α6, in response to injury in a defined culture model [Haq and Trinkaus-Randall, 1998]. These integrin subunits have been shown to play a role in adhesion and migration in both in vivo

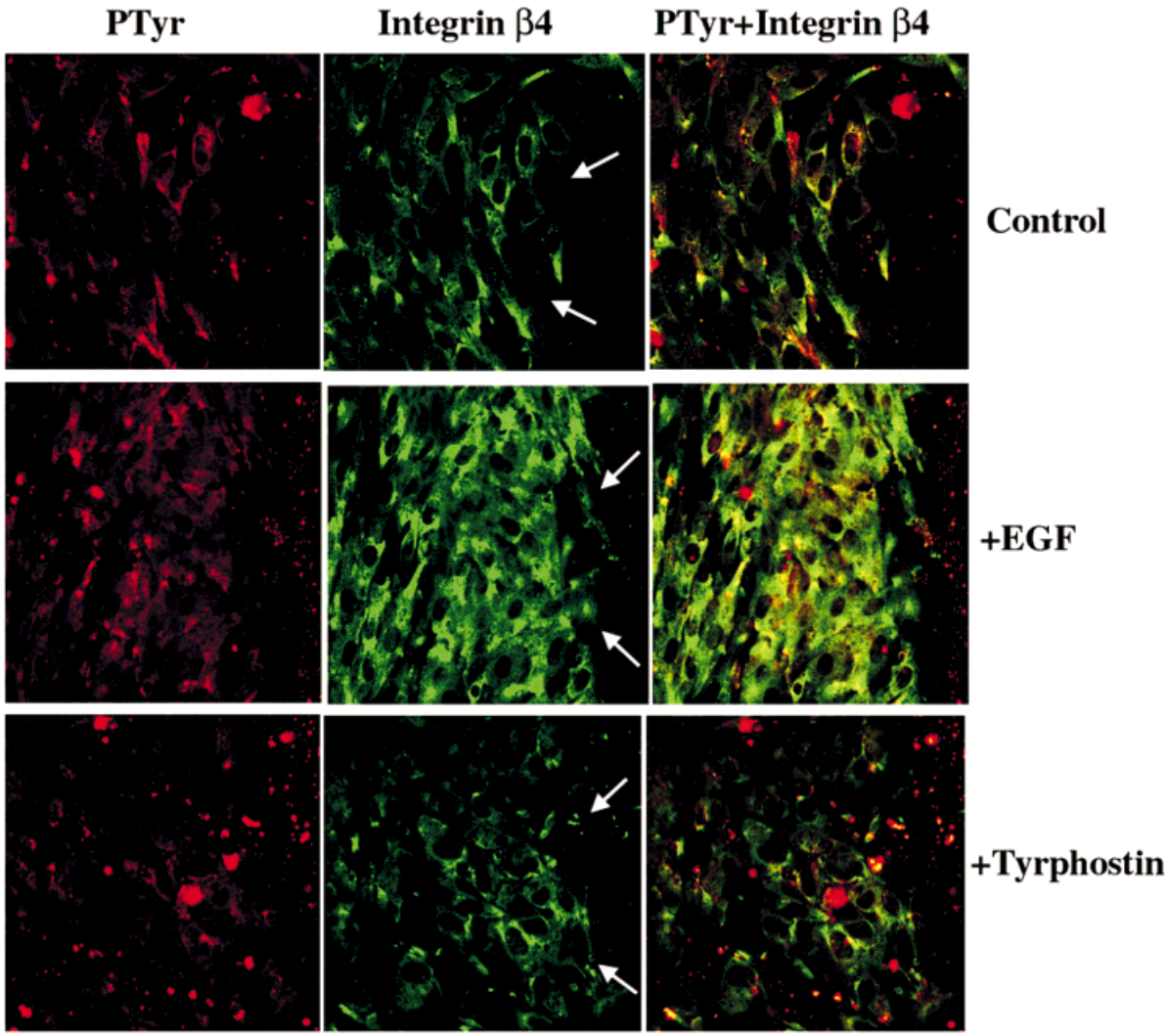

Fig. 9. Expression and activation of β4 integrin subunit are mediated by injury and EGF. Confluent primary epithelial cells were serum-starved, injured, and incubated in the presence or absence of EGF or tyrphostin. The leading edge of the wound is demarcated by arrows. Phosphorylation of β4 was visualized by double staining experiments using a MAb directed against β4 and a polyclonal antibody (ptyr). The data are presented as both separate and merged images with yellow indicating co-localization in the merged image. CLSM imaging programs were used to verify co-localization. β4 expression was present after injury and a number of cells showed co-localization in the cytoplasm. Expression and activation was inhibited with tyrphostin and enhanced with EGF (×196). Experiments are representative of three independent experiments.

and in vitro models. We hypothesized that injury alters the activation of integrin receptors and modulates the availability of EGF that results in changes in receptor occupancy. We found that the localization of specific integrin mRNA changes with injury and that the change is restricted to cells at the wound margin. We also found that the integrin subunit, β4, is activated in response to EGF showing co-localization of ptyr and β4 and that the wound repair rate is facilitated by EGF and inhibited by the inhibitor, tyrphostin, AG1478. These changes in the extracellular environment may trigger alterations in the intensity of the cellular response.

The role of growth factors and their receptors has been studied and the results have given an insight into the mechanisms of wound repair [Clark, 1997]. EGF has been reported to stimulate extensive membrane ruffling, extension of filopodia, retraction of cells from the substratum [Chinkers and Cohen, 1981; Chinkers

et al., 1979], extensive cortical actin polymerization, and depolymerization of actin stress fibers [Schlessinger and Geiger 1981; Rijken et al.,1991]. Both EGF and TGF-α have been shown to promote re-epithelialization of corneal wounds [Schultz et al., 1991]. Epidermal growth factor is a mitogen that binds and activates its cell surface receptor whose cytoplasmic domain is a tyrosine kinase. Once the receptor interacts with the ligand, the EGF receptor phosphorylates a number of proteins including itself. The EGF receptor complexes are internalized via an endocytic pathway [reviews by Schlessinger and Geiger, 1981; Carpenter, 1987; Wells, 1999]. The EGF receptor has been localized in the cornea and shown to vary with age [Zieske et al., 1993, 2000]. There is also evidence suggesting that wound closure that is stimulated by the presence of EGF is correlated with the activation of phosphatidylinositol 3-kinase in immortalized corneal epithelial cells [Zhang and Akhtar, 1997]. This cascade may be stimulated by EGF and may play a role in the proliferation that occurs at the end point of wound closure. Our work on other cells has demonstrated that growth factor receptors are often found at the leading edge of a wound and we hypothesize that this increase in availability for binding may signal the onset of repair events [Song et al., 2000].

Our work has shown that injury alters the expression of β4 mRNA along the wound edge and that this change is enhanced in the presence of EGF. We also demonstrated that injury enhanced both the available binding sites for receptors along the wound margin and tyrosine phosphorylation of the EGF receptor. Both of these occurred rapidly after injury and were negligible within hours after injury. The upregulation of the EGF receptor has been implicated in organ morphogenesis and correlated with invasion of cells [Wells, 1999; Wilson and Gibson, 1999]. Together these results suggest that changes in the environment mediate the regulation of specific integrin subunits that occur in the early events of wound repair.

Many epithelial cell–cell and cell–substrate interactions are mediated through integrins, molecules that are characterized by their ability to dynamically regulate their ligand-binding affinity by transmitting distinct signals from the extracellular matrix to the cell triggering a change in genomic expression. Integrin expression, the affinity and specificity for their ligands, and the integrin–cytoskeleton linkages are regulated by various signals including those initiated by growth factors. In fact, integrins and growth factor receptors share many common signaling events, such as increased tyrosine phosphorylation, activation of mitogen-activated protein (MAP) kinases, protein kinase C isoforms, and small molecular weight GTP-binding proteins, as well as enhanced Ca^{2+} fluxes [Sastry and Horwitz, 1993; Clark and Brugge, 1995, 1996; Yamada and Miyamoto, 1995; Clark and Hynes, 1996; Dedhar and Hannigan, 1996].

Basal epithelial cells adhere to the basal lamina via structures called hemidesmosomes. When the cornea is injured cells migrate to cover the injured area and during this process hemidesmosomes are lost [review by Trinkaus-Randall, 2000]. It has been shown that when this happens the expression of vinculin is elevated while there is a redistribution of bullous pemphigoid antigen [Zieske et al., 1989; Gipson et al., 1993]. Investigators have also demonstrated that the integrin complex, α6β4, is localized to hemidesmosomes [Stepp et al., 1990] and that there are age-related changes that occur along the basal surface of the cells [Trinkaus-Randall et al., 1993]. The coincident expression of α6β4 and laminin by corneal epithelial cells during re-epithelialization suggests that the integrin complex may play a role in mediating epithelial migration [Kurpakus et al., 1991]. In addition others have shown that there was a change in integrin protein with injury [Grushkin-Lerner and Trinkaus-Randall, 1991; Stepp et al., 1996].

Our goal was to define changes in both protein and mRNA in response to injury and EGF. We demonstrated that α6 and β4 mRNA levels increase after injury alone and are accompanied by an increasing number of cells distal to wound edge. In addition both β4 mRNA and protein are enhanced by EGF and while this response is independent of injury, EGF also enhances both the number of cells that respond and the rate of response. These results suggest that a change in α6β4 is critical during wound repair and that responsiveness to changes in the microenvironment are detected as an initial upregulation of cells at the wound edge followed by a decrease as wound closure ensues.

We hypothesize that in vivo injury and the rapid release of peptides from the lacrimal

gland may alter the regulation of integrin receptors resulting in a potentiation of the binding of growth factors to receptors. This would explain the rapid response to injury and change in the bioavailability of growth factors such as EGF. Results from preliminary experiments in organ culture support this hypothesis. In fact this response system can be correlated with disease conditions in other cell types such as epidermis. For example, patients with the skin disorder, psoriasis, are known to overexpress EGF and TGF-α [Elder et al., 1989] and it has also been shown that squamous carcinoma cells overexpress the EGF receptor [Yamamoto et al., 1986]. In both of these the localization of α6β4 is altered and no longer limited to the basal epithelial cells [Pellegrini et al., 1992].

The α6β4 integrin is expressed in a variety of epithelial tissues that is regulated by EGF [Sonnenberg et al., 1990; Kashimata et al., 1997]. Others have demonstrated that EGF induced the migration of cells to laminin and hypothesized that the migration was mediated by α6β4 [Mainiero et al., 1996]. Our results demonstrate that primary epithelial cells will migrate to EGF in a dose-dependent manner in the absence of matrix proteins. However, it is the migration that alters the change in the localization of α6β4. Mainiero et al. [1996] showed that the addition of EGF to cultures resulted in a decrease in immunohistochemical staining of hemidesmosomes in rat bladder 804G cells suggesting that EGF was a factor in the degeneration of the adhesion structure with the onset of migration. They also found that the activation of the EGFR in vitro was coincident with the phosphorylation of β4. Likewise, we have seen that phosphorylation of the β4 subunit can be inhibited in the presence of tyrphostin. This observation has also been seen with the insulin receptor [Shechter et al., 1989]. However, phosphorylation of β4 is not associated with degeneration and the complete formation of hemidesmosomes requires phosphorylation of β4 and BPAG-1 [Payne et al., 2000].

To evaluate the role of EGF in the wound cultures, cells were incubated in the presence or absence of tyrphostin. We demonstrated that injury enhanced the expression of EGF receptors and induced a transient increase in the availability of EGF binding along the wound edge. These results contrasted with those of Takahashi and Suzuki [1996] who did not detect any significant difference in binding. However, the differences may likely be explained by an averaging of the signal, as we have diminished signal if our analysis is only in the form of analysis of extracts and is not correlated with immunohistochemical evaluation [Song et al., 2000]. This is likely as the EGF receptor was localized along the outer edge of islands of low density cells. Change in expression of receptors with cell density has been speculated to simulate injury by other researchers [Richardson et al., 1999]. The EGF receptor is a transmembrane protein of 170 kDa that catalyzes EGF-dependent tyrosine phosphorylation of various proteins as well as autophosphorylation of the receptor [Ullrich et al., 1984]. Studies have shown that activation of the EGF receptor leads to increased cell motility [Blay and Brown, 1985; Chen et al., 1993, 1994a,b; Klemke et al., 1994; Fujii et al., 1995; Matthay et al., 1993; Basson et al., 1992] and production of ECM degrading proteases [Yoshida et al., 1990; Rorth et al., 1990; Niedbala et al., 1990; Kessler and Markus, 1991], thereby supporting a role for the EGF receptor in both normal development and pathological processes.

Tyrphostins are known to inhibit kinase activity of EGFR in vitro [Lyall et al., 1989]. We demonstrated that it inhibits cell migration extensively at 1 μM in our injury model. While significantly higher concentrations of tyrphostin were required for inhibition in the organ culture experiments, our results support those of Zieske et al. [2000] who demonstrated that the receptor is activated and that the localization of the receptor changes with migration of cells in response to injury. The use of tyrphostin allowed us to distinguish between the activation of the receptor and binding as it does not inhibit EGF binding or internalization and degradation of both receptor and EGF. Given these observations, our next goal was to determine if the expression of β4 in response to injury and EGF occurred via the activation of EGFR. Phosphorylation of β4 was detected in the presence of exogenous EGF and less so in response to injury alone. We also have evidence demonstrating that β4 is phosphorylated during the formation of complete hemidesmosomes in organ culture and that phosphorylation of β4 is reduced when hemidesmosome formation is compromised [Payne et al., 2000]. Additional experiments where phosphorylation of tyrosine

residues was inhibited resulted in lack of hemidesmosome formation in an organ culture system where epithelial sheets are incubated on substrata containing an intact basal lamina. These observations may merely indicate that the process of phosphorylation of β4 can occur via distinct pathways. These results demonstrate that cellular responsiveness to changes in the environment may provide coordinated EGF binding and integrin expression at different stages in the wound repair process. These mechanisms may have a particular role in the cornea that is avascular and where changes in the bioavailability of growth factors are uniquely controlled.

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