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Dynamic Characterization of the Molecular Events During *In Vitro* Epidermal Wound Healing

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The aim of this study was to characterize some of the molecular events stimulated *in vitro* in response to injury within a confluent culture of normal epidermal keratinocytes as a model to understand the mechanisms of wound healing. To this end, an original device was developed specifically designed to perform calibrated injuries of great lengths within mono-stratified or pluri-stratified keratinocyte cultures. The experiments performed in this study validate this device as an appropriate tool for studying epidermal wound healing; this is because it performs mechanical injuries that stimulate the expression of multiple healing markers also known to be upregulated during wound healing *in vivo* (growth factors, cytokines, proteinases, extracellular matrix proteins). Using this device, it was demonstrated in human keratinocytes: mechanical injuries (i) immediately stimulate the tyrosine phosphorylation of numerous cellular proteins; (ii) induce molecular cascades leading to the activation of p21ras, mitogen-activated protein kinases, extracellular signal-regulated kinases 1/2, c-Jun NH2 terminal kinase, and p38 mitogen-activated protein kinase; and (iii) increase the phosphorylation of their respective substrates, c-jun and activator transcription factor 1. Wounding of these cells also results in increases in the DNA binding activities of several jun/fos activator protein-1 transcription factor complexes. It is important to note that the development of an appropriate wounding system was essential for performing this study, as use of a classical wounding procedure did not enable the detection of the biologic parameters reported above. In conclusion, these data indicate that using the appropriate system, it is possible to identify the signaling pathways activated in normal human keratinocyte cells after injury. In this study, it was shown that the mitogen-activated protein kinase pathways and activator protein-1 are stimulated in response to physical injury, and may be involved in regulating the expression of healing markers.* ***Key words: keratinocytes/metalloproteinases/mitogen-activated protein kinases/phosphotyrosine/wound healing. J Invest Dermatol 119:56–63, 2002***

Skin wound healing is a complex process that involves inflammation, re-epithelialization, neoangiogenesis, and connective tissue cell activation with subsequent extracellular matrix (ECM) degradation and resynthesis (Clark, 1996; Bello and Phillips, 2000). All these processes are regulated by cell/ECM interactions and by cytokines and/or growth factors. In skin cells, injury is likely to initiate a series of changes in gene expression that mediate the downstream events required for tissue repair, including cell proliferation, migration, and remodeling of the extracellular matrix (Martin, 1997); however, understanding of the different wound signal transduction pathways remains poorly documented. Among them, the mitogen-activated protein kinases (MAPK) pathways are suspected to participate in the healing process (Jaakkola *et al*, 1998), but their activation and involvement in this process has yet to be concretely demonstrated.

The MAPK pathways have been established as the major signaling modules through which cells transduce extracellular signals. Three distinct MAPK cascades have been identified: (i) extracellular signal-regulated kinases (ERK); (ii) c-jun-N-terminal protein kinase; and (iii) p38[MAPK]. Activation of ERK is thought to play an important part in mediating cell proliferation in response to growth factors. JNK and p38[MAPK] are activated mostly by cellular stresses but also by mitogens. Activation of the appropriate cell surface receptors results in the activation of downstream components of the cascades, including Rho GTPase family members, and ultimately leads to the phosphorylation of nuclear transcription factors (Karin, 1998). The balance between the activation of one or more of these pathways and the duration of the activation period are believed to be critical in determining cell phenotype and the nature of the activated downstream genes. For

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Abbreviations: NHK, normal human keratinocytes; ECM, extracellular matrix; uPA, urokinase-type plasminogen activator; HB-EGF, heparin-binding epidermal growth factor-like; RPA, RNase protection assay; AP-1, activator protein-1; ERK, extracellular signal-regulated kinase; jun kinase/JNK, c-jun NH2 terminal kinase; p38[MAPK], p38 mitogen-activated protein kinase.

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this reason, we aimed to characterize the early signaling events that are triggered during wound healing in normal human keratinocytes (NHK). These results will ultimately improve our understanding of skin wound healing at the molecular level. Several *in vitro* wounding systems already exist (Herman, 1993; Dieckgraefe *et al*, 1997; Goke *et al*, 1998; Chen *et al*, 2000); however, none of them have been proven to be efficient at detecting the molecular events activated during wound healing in keratinocytes. For this reason, we have developed a new system that enables the detection of a wide spectrum of molecular events activated during *in vitro* wound healing. Using this system, it was demonstrated that the injury of a keratinocyte cell culture provokes the activation of early signaling events, including tyrosine kinases, MAPK, transcription factors, and the subsequent upregulation of several wound healing markers known to be stimulated during wound healing *in vivo*.

MATERIALS AND METHODS

Reagents Trypsin, ethylenediamine tetraacetic acid, HEPES, penicillin, and streptomycin were purchased from Life Technologies (Life Technologies, In Vitrogen Corp., Carlsbad, CA). Dulbecco's minimal Eagle's medium, Ham F12, and fetal bovine serum were obtained from Hyclone (Hyclone, Northumberland, UK). Insulin, hydrocortisone, cholera toxin, human recombinant epidermal growth factor (EGF), triiodothyronine, adenine, mitomycin, gelatin, and H-D-Val-Leu-Lys-*p*-nitroanilide were purchased from Sigma (Sigma, Aldrich, St Louis, MO). Bromodeoxyuridine (BrdU) was provided by Amersham (les Ulis, France). RNABle, RNA preparation kit was from Eurobio (Eurobio, les ULIS, France). Plasminogen was purchased from Calbiochem (Calbiochem/France Biochem, Meuden, France). B428 (Towle *et al*, 1993), was a generous gift of Dr G. Kuznetsov (Eisai Laboratories Andover MA).

Isolation and primary culture of human keratinocytes

Isolation Human keratinocytes were isolated from healthy neonatal foreskin as described (Rheinwald and Green, 1975).

Cell culture Keratinocytes were seeded on mitomycin C (10 μg per ml) treated 3T3-J2 fibroblast feeder layers (2×10^5 cells per cm^2) and grown in "Green" medium (2/3 Dulbecco's minimal Eagle's medium, 1/3 Ham F12 supplemented with 10% fetal bovine serum, 20 mM HEPES, 1000 U penicillin per ml, 1000 U streptomycin per ml, 5 μg insulin per ml, 0.4 μg hydrocortisone per ml, 10 μM cholera toxin, 10 μM recombinant human EGF, 2 μM triiodothyronine, and 18 μM adenine.

Antibodies The monoclonal anti-phosphotyrosine (4G10) and anti-BrdU antibodies were obtained from Upstate Biotechnology Inc. (New York) and from Roche/Amersham, respectively. Polyclonal anti-ACTIVE ERK1/2, JNK (pTPpY), $p38^{[MAPK]}$ (pTPpY) antibodies were purchased from Promega (Madison, WI). The phospho-specific c-Jun (Ser^{63} and Ser^{73}) and CREB (Ser^{133}) antibodies were from Biolabs (New England Biolabs Inc. Beverly, MA). The monoclonal anti-Ras antibody (anti-pan-Ras) was from Calbiochem. Horseradish peroxidase-conjugated goat anti-rabbit IgG and horseradish peroxidase-conjugated rabbit anti-mouse IgG were purchased from Dako (DAKO, Copenhagen, Denmark).

Probes The cDNA encoding the human *c-jun*, *c-fos*, and *junB* were a gift of Dr. J.C. Chambard (Nice, France). The cDNA probe corresponding to urokinase-type plasminogen activator (uPA) was a gift of Dr. A. Schmid-Alliana (Nice, France). The full-length heparin-binding epidermal growth factor-like (HB-EGF) cDNA was provided by Dr. B. Mograbi. The matrix metalloproteinase (MMP)-9 (458 bp) and the fibronectin External Domain A (EDA) 250 bp cDNA probes were obtained by polymerase chain reaction amplification from NHK RNA using specific primers, respectively: (sense: 5′-TGGCCCAGGTGACC-GGGGCCCTC-3′; anti-sense: 5′-AAAGGTTAGAGAATCCAAGTT-TAT-3′) and (sense: 5′-AACATTGATCGCCCTAAAGGACT-3′; anti-sense: 5′-TGTGGACTGGGTTCCAATCAGGGG-3′). The integrin β4 cDNA (1.9 kb) and the full-length cDNA encoding laminin-5 α3a, laminin-5 β3, and laminin-5 γ2 were a gift of Dr. Y. Gache (Nice, France).

Cell proliferation Confluent NHK cells grown in complete "Green" medium were wounded with a sterile blade and incubated at 37°C/5% CO_2 for the indicated times. Four hours before the end of the incubation, 10 μM BrdU was added to the cell culture medium. The cells were washed with phosphate-buffered saline and fixed in 1% paraformaldehyde. The BrdU incorporated into the DNA was revealed, as previously described (Brunet *et al*, 1999), using a specific monoclonal antibody.

Protein extracts and immunoblotting analysis Cells were wounded for the indicated times (control cells were left unwounded), and solubilized at 4°C in 500 μl RIPA buffer (Tris–HCl 10 mM pH 7.5, 150 mM NaCl, sodium deoxycholate 1%, Nonidet P-40 1%, sodium dodecyl sulfate 0.1%, 1 mM phenylmethylsulfonyl fluoride, 50 μg aprotinin per ml, 50 μg leupeptin per ml, 1 μg pepstatin A per ml, 20 mM NaF, 1 mM $NaVO_4$, 1 mM β glycerophosphate). Proteins (100 μg per lane) were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred on to Immobilon-p membrane (Millipore, Bedford, MA) as described previously (Turchi *et al*, 2000). The membrane was saturated with phosphate-buffered saline supplemented with 3% bovine serum albumin, 0.5% gelatin, and 0.1% Tween 20, and incubated with either anti-active ERK (1/4000), anti-active JNK (1/5000), anti-active $p38^{[MAPK]}$ (1/2000), or anti-PY (1/4000), for 2 h at room temperature. The membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (1/10000) or rabbit anti-mouse IgG (1/5000) and blots were revealed using the Amersham ECL detection system.

P21ras activation (pull-down experiments) P21ras activation was measured by precipitation with the GST fusion protein containing the Ras binding domain of Raf-1 (RDB) (Herrmann *et al*, 1995). Briefly, wounded (or control) keratinocytes were solubilized in lysis buffer (50 mM Tris–HCl pH 7.5, 15 mM NaCl, 20 mM $MgCl_2$, 5 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, Triton X-100 1%, *N*-octylglucoside 1%, 100 μM AEBSF, 100 μM leupeptin, 1 μM Pepstatine A). One milligram of clarified protein extract was incubated with glutathione-Sepharose-GST-RBD protein (30 μg) for 2 h at 4°C. The beads were washed four times with buffer, denatured in 30 μl of Laemmli sample buffer and p21ras was analyzed by immunoblotting using the monoclonal antibody described above.

Immune complex kinase assay This technique, used to measure JNK and $p38^{[MAPK]}$ activities, was described previously (Turchi *et al*, 2000).

Northern blot analysis Total RNA from NHK were isolated using the RNAble kit according to the manufacturer's instructions. Northern blot analysis were carried out using 20 μg of total RNA following the procedure described previously (Rezzonico *et al*, 1995).

RNase protection assay NHK mRNA were prepared and extracted as described above and hybridized using a Multiprobe RNAse protection assay (RPA) system following manufacturer's directions (Pharmingen Becton Dickinson, Palo Alto, CA). Briefly, 10 μg of total RNA were hybridized with a ^{32}P-labeled hCK-3 or hCK-5 template set (6×10^5 cpm per sample) for 16 h at 56°C, then sequentially treated with RNase A + T1 and proteinase K. After phenol extraction and ethanol precipitation, the samples were run on a 5% acrylamide/bis acrylamide (29:1) urea containing gel, dried, and autoradiographed.

Reverse transcriptase–polymerase chain reaction Total NHK RNA (2 μg) was reverse-transcribed at 37°C for 60 min, using Omniscript Kit (Promega). Five microliters of each cDNA sample were used for polymerase chain reaction using TAQ polymerase (Life Technologies). The cDNA encoding laminin-5, fibronectin (EDA), and HB-EGF were amplified using the specific sets of primers described above. The oligonucleotides used for the amplification of granulocyte-macrophage-colony stimulating factor cDNA were: forward, 5′-GAG-CATGTGAATGCCATCCAGGAG-3′, reverse, 5′-CTCCTGGACTG-GCTCCCAGCAGTCAAA-3′.

Gelatin zymography Culture medium (10 μl) or cell lysates (20 μg) from wounded or intact NHK were analyzed on 10% polyacrylamide gel containing 0.3% gelatin. The gel was incubated in 2.5% Triton X-100 for 1 h and placed at 37°C in 30 mM Tris–HCl, pH 7.5200 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ for 18 h. The gel was finally stained with Coomassie Brilliant Blue.

UPA enzymatic activity Cell culture medium (25 μl) or cellular extracts (2.5 μg) were incubated for 30 min at 37°C with plasminogen (0.03 μM) in 10 mM Tris–HCl pH 7.6, 50 mM NaCl. The chromogenic substrate, H-D-Val-Leu-Lys-p-nitroanilide (200 μM), was added and the absorbance of the cleavage products was measured at 410 nm using a Dynatech MRX microplate reader.

Electrophoretic mobility shift assay NHK were lyzed in Totex lysis buffer (20 mM HEPES, pH 7.9, 350 mM NaCl, 20% glycerol, 1%

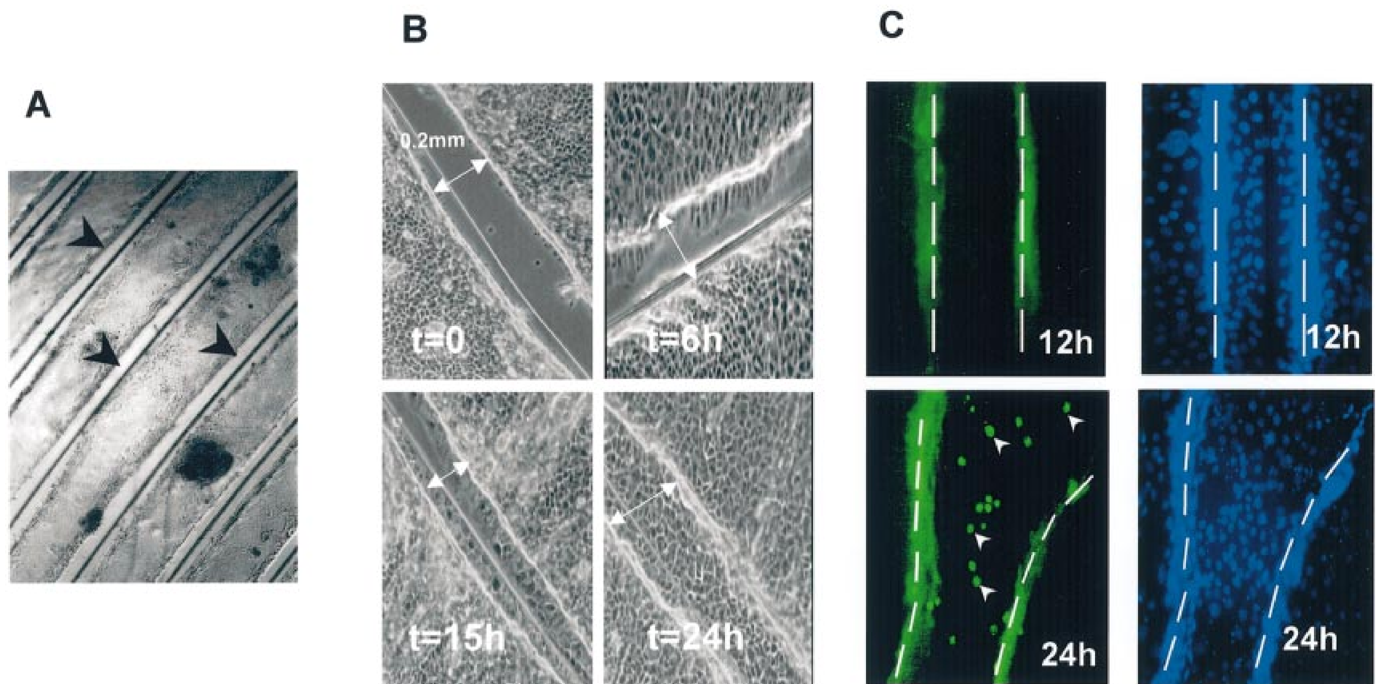

Figure 1. Migration and proliferation of KHN in response to injury. (*A*) Morphology of a typical "wounded" NHK culture (original magnification × 50). The device creates a series of parallel circular "grooves" (*black arrows*) (six grooves shown here) separated by nonwounded regions. (*B,C*) Kinetics of wound healing after mechanical injury. Confluent cultures of NHK, deprived of serum and EGF for 18 h, were wounded with a sterile blade, and the recolonization was studied for 6 h or 12 h and 24 h (*A*) by light microscopy (original magnification × 200) the white arrows indicates the "wound bed") (*B*) by immunofluorescence (original magnification × 200) after BrdU staining (green fluorescence). The arrows indicate (some of) the BrdU-labeled nuclei in the wound bed. The dotted lines represent the wound edges. The blue fluorescence (right part) shows the cell nuclei stained with Hoechst 3358 reagent.

Nonidet P-40, 1 mM $MgCl_2$, 0.5 mM ethylenediamine tetraacetic acid, 0.1 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, 0.5 mM dithiothreitol, 0.1% phenylmethylsulfonyl fluoride, and 0.1% aprotinin) according to Baeuerle and Baltimore (1988). *In vitro* binding reaction was performed in 25 μl of binding buffer [10 mM HEPES, pH 7.8, 50 mM KCl, 2 mM dithiothreitol, 1 mM ethylenediamine tetraacetic acid, 5 mM $MgCl_2$, 10% glycerol, 3 mM AEBSF, 2 mg poly(dI-dC) per ml and 2 mg bovine serum albumin per ml] by incubating 5 μg of whole cell extracts with 2×10^5 cpm of ^{32}P-end–labeled oligonucleotide probes. After 20 min at 25°C the DNA/protein complexes were separated by electrophoresis on polyacrylamide gel (4%) in TAE for 1 h at 300 V. The gel was dried and exposed on autoradiography film. For antibody supershift assays, nuclear extracts were preincubated for 30 min at 4°C with 0.3 μl of anti-sera raised against different members of the Jun and Fos families (Lallemand *et al*, 1997), before the addition of the labeled DNA probes.

RESULTS

A new device to investigate the molecular events related to the wound healing process We have developed a novel device designed specifically to investigate the molecular mechanisms involved in wound healing. This device performs, in confluent cell cultures, a continuous curvilinear wound whose length reached 15–20 meters when performed on 100 mm diameter (55 cm^2) culture dishes (**Fig 1*A***). The wound length and, therefore, the number of cells involved in the wound healing process can be finely regulated. Our experiments indicate that in response to a typical wound performed using this apparatus, about 40% of the total cell population are involved in the healing process. In addition, this device may be equipped either with a scalpel blade or a laser optical fiber and thus can generate mechanical or thermal wounds. The principle and the technical characteristics of this invention are covered by a patent (FR 0108088).

The results presented in **Fig 1** show that the mechanical wounding of a confluent NHK cell culture (induced by a scalpel blade) generated a 200–300 μm wide "wound bed" that was spontaneously filled within hours following injury. The chronology of these "healing" events was in accordance with previously reported data (Krawczyk, 1971; Ortonne *et al*, 1981; Garlick and Taichman, 1994). The "healing" process began 6 h after the injury with the migration of cells situated at the wound margin (**Fig 1*A***). The proliferation burst (which follows the initial migration), assessed by the incorporation of BrdU, occurred 24 h after injury (**Fig 1*B***). As shown in panel A, the "wound bed" was completely covered after 24 h.

Validation of the wounding system To verify the biologic relevance of this wounding system, we studied its effect on the expression of a wide range of genes reported to be regulated at the wound edge of keratinocytes during *in vivo* wound healing. The list includes: (i) early genes (*c-fos, c-jun*, and *junB*) (Marshall *et al*, 1992; Martin and Nobes, 1992); (ii) growth factors and cytokines (such as HB-EGF; Marikovsky *et al*, 1993); (iii) interleukin (IL)-1 (Sauder *et al*, 1990; Komine *et al*, 2001); (iv) transforming growth factor (TGF)-β (Kane *et al*, 1991; Matsumoto *et al*, 1997); (v) IL-8 (Rennekampff *et al*, 2000), or granulocyte-macrophage-colony stimulating factor (Park *et al*, 2001); (vi) extracellular matrix components (laminin-5 α3, β3, γ2; Larjava *et al*, 1993; Kainulainen *et al*, 1998; Nguyen *et al*, 2000); (vii) fibronectin EDA, a spliced species of fibronectin specifically induced during wound healing (Manabe, 1999; French-Constant, 1989); (viii) β4 integrin (Cavani *et al*, 1993; Donaldson *et al*, 1995; Goldfinger and Hopkinson, 1999); (ix) protease activities (MMP-9, MMP-2; Salo *et al*, 1994; Madlener *et al*, 1998); and (x) uPA (Grondahl-Hansen *et al*, 1988; Romer *et al*, 1991). Confluent NHK grown in 100 mm culture dishes were wounded as described in *Materials and Methods* and harvested after 1, 3, 6, 15, 24, 48, and 72 h, and the expression of the molecules mentioned above was analyzed using several techniques (northern blot, RPA, polymerase chain reaction, enzyme-linked immunosorbent assay, and zymography).

The results presented in **Fig 2(*A*)** clearly demonstrate that *c-jun* and *c-fos* mRNA were strongly stimulated 30 min and 1 h after injury, respectively. This is not the case for *JunB* mRNA, which was constitutively expressed and did not increase during healing.

We also analyzed the expression of growth factors and cytokines known to be upregulated *in vivo* during wound healing. In accordance with data previously reported in the literature (see References above), we have observed using northern blotting, RPA, and polymerase chain reaction, that expressions of HB-EGF, IL-8, and granulocyte-macrophage-colony stimulating factor mRNA were markedly increased between 4 and 24 h after mechanical injury (**Fig 2*A*, *C***). The data presented in **Fig 2(*B*)** indicate that the three TGF-β isoforms (TGF-β1, TGF-β2, TGF-β3) were expressed in cultured keratinocytes. When the cell culture was wounded, TGF-β1 and TGF-β3 increased, whereas the levels of TGF-β2 did not vary. The levels of TGF-β1 and TGF-β3 reached a maximum at 6 h after wounding then decreased rapidly. As illustrated in **Fig 2(*D*)**, IL-1α and IL-1β, measured by enzyme-linked immunosorbent assay (Ferrua *et al*, 1988) were also strongly stimulated during the cell repair process. The stimulation (more than 100-fold over the basal levels) was detectable after 4 h and lasted at least up to 24 h post-wounding (not shown).

Proteinases and components of the ECM play an essential part in tissue remodeling and cell migration and thus are key regulators of wound healing *in vivo*. To analyze the behavior of these classes of molecules in our *in vitro* system, we have tested the effects of mechanical injury on the levels of: (i) the proteinases MMP-9, MMP-2, and uPA; (ii) laminin-5 (α3, β3, and γ2 subunits), and fibronectin EDA; and (iii) the β4 integrin subunit, the signal transducing moiety of the laminin-5 receptor, α6β4 (Niessen *et al*, 1994). As shown in **Fig 3**, all these markers of wound regeneration were stimulated *in vitro* in response to mechanical injury performed with our ''wounding apparatus''. The proteases (generally involved in cell migration) were induced first (3 and 10 h ''postwounding'' for uPA and MMP-9, respectively) (**Fig 3*A***), whereas ECM proteins and integrin β4 (proteins participating in cell adhesion) were increased significantly 24 h after injury (**Fig 3*B***) when the wound bed was totally recovered. The data presented in **Fig 4** clearly show that uPA and gelatinase B mRNA stimulation was associated with the increase in the soluble and cell-associated active enzymatic forms of these proteins (**Fig 4*A*** and **4*B***, respectively).

To justify the necessity for developing a new *in vitro* wounding system different from that already used to describe healing (Dieckgraefe *et al*, 1997; Goke *et al*, 1998; Pintucci *et al*, 1999), we have compared the expression of (i) MMP9 and laminin-5 (β3 and γ2 subunits) mRNA (15 h postwounding), and (ii) IL-1α and IL-1β proteins (5 h postwounding) in NHK cultures wounded either with our system or with a classical laceration technique (50 parallel streaks carried out with a scalpel blade). Results depicted in **Fig 5(*A*, *B*)** clearly demonstrate that these biologic parameters

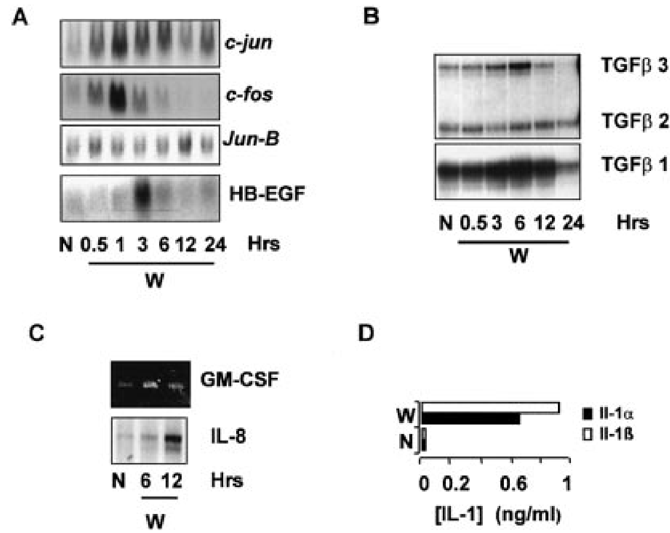

Figure 2. Expression of specific mRNA and IL-1 secretion in wounded NHK cell cultures. Fetal bovine serum/EGF starved confluent NHK cells were wounded (W) or not (N). At indicated times, culture media was collected and total RNA was prepared from the cells using the RNABle® kit. (*A*) HB-EGF, *c-jun*, *junB*, and *c-fos* mRNA were analyzed by northern blot using specific probes radiolabeled by random priming. (*B*) TGF-β1, TGF-β2, and TGF-β3 mRNA were analyzed by RPA as described in *Materials and Methods*. (*C*) IL-8 and granulocyte-macrophage-colony stimulating factor mRNA were analyzed, respectively, by RPA and polymerase chain reaction. (*D*)IL-1α and IL-1β secreted in the medium of wounded cultures were measured by enzyme-linked immunosorbent assay.

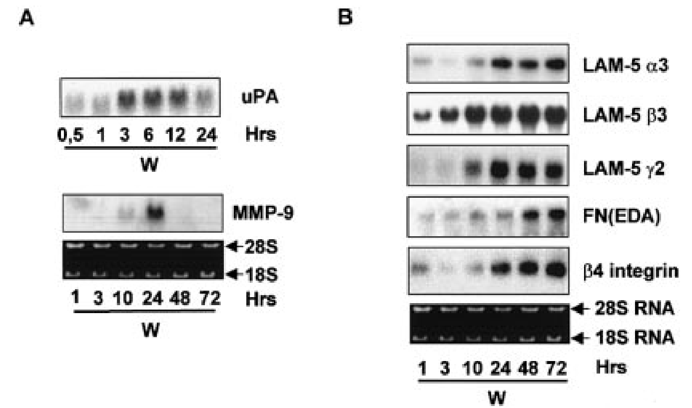

Figure 3. Effect of mechanical injury on the expression of ECM proteins and proteolytic activities. Confluent NHK cultures were deprived of serum and EGF for 18 h and injured (W) or not (N) with our wounding system. At the indicated times total RNA was extracted and uPA and MMP-9 expression were measured by northern blot (*A*). (*B*) Northern blot expression of ECM proteins and integrin β4 mRNA in ''healing'' cells. LAM 5 α3, β3, γ2 correspond, respectively, to the α3, β3 and γ2 subunits of laminin-5; fibronectin (EDA) corresponds to the EDA spliced form of fibronectin. Ethidium bromide labeled 18S and 28S RNA are shown as loading controls.

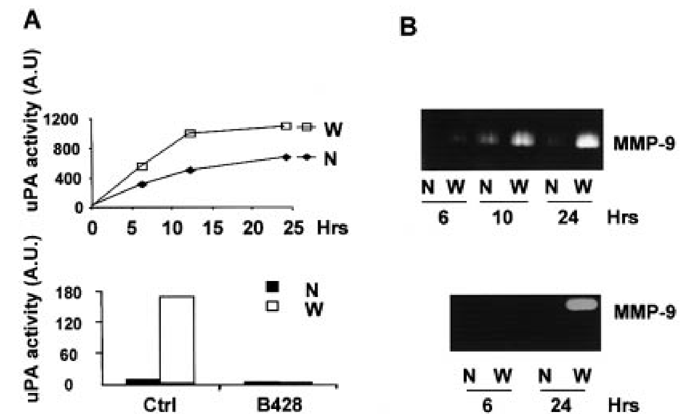

Figure 4. Measurement of MMP-9 and uPA enzymatic activities. (*A*) UPA activity was determined in the culture medium (upper part) of intact (N) or wounded (W) NHK cells, using a colorimetric test with H-D-Val-Leu-Lys-p-nitroanilide as substrate. The lower part represents the cell-associated activity, measured before (■) and 4 h after injury (□), in the presence or absence of 10 μM of B428, a pharmacologic inhibitor of uPA. (*B*) MMP-9 activity was estimated by zymography (showing gelatinase activity) of the culture medium (upper part) and lysates (lower part) of intact (N) or wounded (W) NHK cells. The data shown are representative of three independent experiments.

were hardly detectable using the classical laceration procedure, whereas they were strongly upregulated when the NHK were wounded using our device.

In summary, these data show that this *in vitro* wounding model represents a biologically relevant system to study the molecular mechanisms responsible for epidermal wound repair.

Mechanical injury of a NHK cell culture stimulates tyrosine phosphorylation and MAPK pathways We next focused on the early molecular events induced by "wound healing" *in vitro*. The first step of this study was to measure the level of tyrosine phosphorylation in cell lysates obtained from wounded and nonwounded NHK. As shown in **Fig 6(*A*)**, wounding rapidly stimulated the tyrosine phosphorylation of several proteins whose molecular weights corresponded to 135, 125, 117,112, 95, 84, 64, 60, 56, 53, 39, and 36 kDa. The phosphorylation induction was rapid and transient, reaching a maximal value at 8 min postwounding.

Next we examined the effects of mechanical injury on the MAPK pathways, known to regulate many biologic functions, including proliferation, differentiation, and apoptosis. To this end, the activation of ERK1/2, Jun kinase, and $p38^{[MAPK]}$ were measured by western blot using antibodies raised against the phospho-active forms of each kinase. The results shown in **Fig 6(*B*, *C*)** indicate that the ERK1/2 (**Fig 6*B***), $p38^{[MAPK]}$, and JNK pathways (**Fig 6*C***) were strongly activated when a confluent culture of NHK cells was wounded. The stimulation was rapid (8 min); however, whereas the JNK and $p38^{[MAPK]}$ activation was transient, the ERK1/2 stimulation was biphasic, rising to maximal levels after 15 min and then at 5 h postwounding (**Fig 6*B***). Interestingly, ERK, JNK, and $p38^{[MAPK]}$ were only activated when NHK cultures were wounded using our device but not with the classical laceration technique (50 parallel streaks) (data not shown), suggesting that a correlation might exist between the activation of these signal transducers and the induction of the healing markers described above (**Fig 5**).

The pull down experiments depicted in **Fig 6(*B*)** demonstrate that injury of confluent NHK stimulated the activation of the p21ras oncogene, which is a known upstream component of ERK1 activation within 5 min. Finally NHK injury induced the phosphorylation of c-Jun and activator transcription factor 1, two physiologic substrates of JNK and $p38^{[MAPK]}$, respectively (**Fig 6*C***). The phosphorylation was detected after 20 min and lasted at least until 60 min postwounding.

NHK cell culture injury stimulated activator protein (AP)-1 The AP-1 family of transcription factors plays a dominant part in keratinocyte differentiation *in vivo* during trauma and wound healing, where it regulates the expression of genes such as laminin-

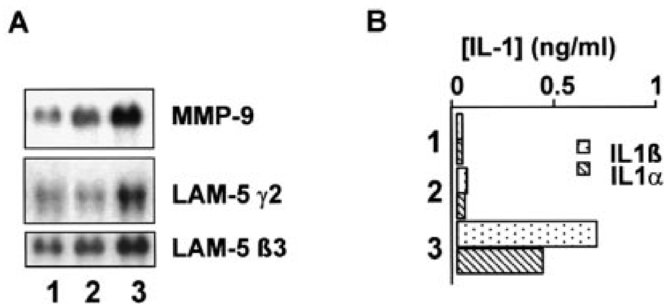

Figure 5. Comparison between classical scalpel injury and mechanical injury induced by our apparatus. Confluent and serum starved NHK cells were wounded either by 50 parallel streaks carried out with a scalpel blade (classical laceration technique) (2), with our automated device (3), or kept intact (1), then different specific biologic parameters were assayed. (*A*) mRNA levels of laminin-5 β3 and γ2 subunits (LAM-5 β3, γ2) and MMP-9 performed by northern blot. Panel (*B*) depicts the IL-1 α and β production measured by enzyme-linked immunosorbent assay.

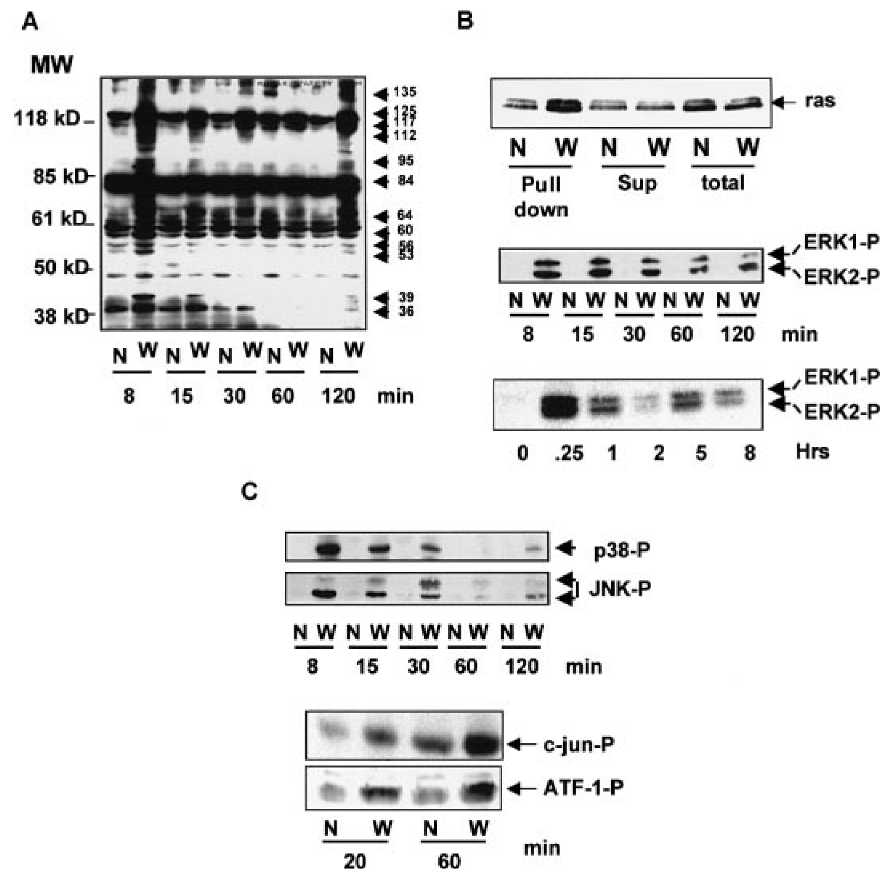

Figure 6. Effect of mechanical wounding on tyrosine phosphorylation, Ras activation, and MAPK activities. Fetal bovine serum and EGF starved NHK cell cultures were wounded (W) and then replaced at 37°C for the indicated times. The cells were solubilized and the lysates were analyzed by western blot using specific antibodies, or treated for analysis of Ras activation by pull down assays as described previously. (*A*) Represents the pattern of tyrosine phosphorylated proteins determined using the 4G10 monoclonal antibody. The arrows indicate the apparent molecular weight of the different phosphoproteins. (*B*) GST-RDB precipitated p21ras and ERK 1/2 activities were analyzed using a monoclonal anti-ras antibody or antibodies raised against the active/phosphorylated forms of ERK 1/2, respectively. (*C*) Activated JNK 1/2, $p38^{MAPK}$ and phosphorylated c-jun and activator transcription factor 1 were assayed using antibodies raised against the active/phosphorylated forms of each protein.

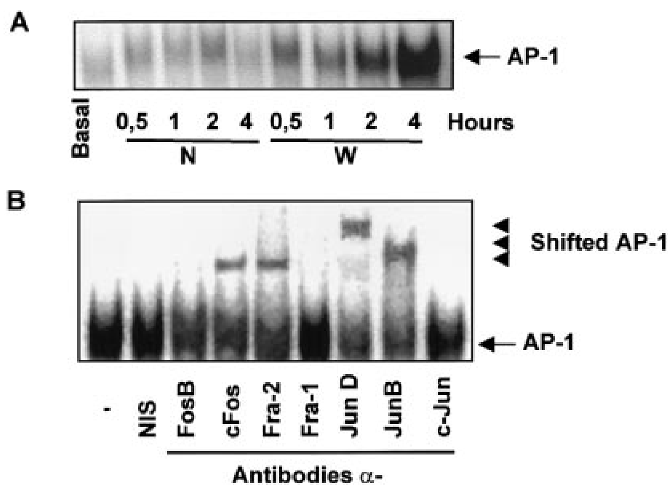

Figure 7. AP-1 activation in response to mechanical injury. Confluent, serum- and EGF-starved NHK cells were wounded (W) or not (N) and harvested at the indicated times. Nuclear extracts were prepared as described in *Materials and Methods*, then AP-1 DNA-binding activity was measured by electrophoretic mobility shift-assay (EMSA) performed with a specific ^{32}P labeled TRE consensus sequence (*A*). (*B*) Characterization of AP-1 complexes induced by NHK injury (supershift experiments). Nuclear extracts were prepared from cells 4 h postwounding and then supershift experiments were performed by preincubating nuclear proteins either with nonimmune serum (NIS) or specific polyclonal antibodies as described in *Material and Methods*.

5, matrix remodeling metalloproteinases, keratins, TGF-β, or syndecan-1 (Navarro *et al*, 1995; Rosenberg, 1995; Jaakkola *et al*, 1998; Virolle *et al*, 1998; Verrecchia *et al*, 2001). The exact composition of the different complexes upregulated during wound healing is still unknown, however.

We have measured the DNA binding activity of AP-1 in NHK cultures before and after injury. Confluent NHK cultures were harvested at 0.5, 1, 2, and 4 h after injury, nuclear extracts were prepared and the DNA binding activity was examined by gel mobility shift assays using a consensus labeled target sequence for AP-1 (TRE) (Virolle *et al*, 1998). Results in **Fig 7(*A*)** indicate that AP-1 complexes were stimulated exclusively in injured cells. The stimulation was visible 2 h after wounding and peaked at 4 h, which is in accordance with previously published observations (Gallucci *et al*, 2000).

The broad amplitude of AP-1 stimulation prompted us to identify the different Fos/jun members of the AP-1 complex induced in healing NHK (Lallemand *et al*, 1997). To this end, nuclear extracts from regenerating cultures were subjected to supershift experiments in gel retardation assays using specific anti-jun and anti-fos antibodies. Nuclear extracts of cells at 4 h postwounding were preincubated either with preimmune IgG or with antibodies specific for c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB, and JunD, before analysis of AP-1 DNA-binding activity using a TRE consensus sequence. The results (**Fig 7*B***) showed that anti-c-fos, Fra-2, FosB, JunB, JunD, and, to a lesser extent, anti-c-Jun induced a supershift and/or an inhibition of the DNA-binding activity of the complex. These data demonstrate for the first time that the binding activity of at least six distinct types of AP1 complexes, i.e., c-Fos/JunB, c-Fos/JunD, Fra-2/JunB, Fra-2/JunD FosB/JunD, and FosB/JunB heterodimers, is increased after injury in NHK culture.

DISCUSSION

The cutaneous wound healing process is a complex phenomenon characterized by the onset of a series of molecular events with a great degree of cross-talk (Singer and Clark, 1999). Today, the identification of these different pathways is a matter of great interest for both the characterization of the physiologic abnormalities leading to aberrant skin repair in acquired or inherited wound healing disorders, and for the improvement of current pharmacologic treatments.

This study was begun in an effort to progress in this direction. By means of a novel cell wounding system, specially designed for this purpose, we have identified in normal epidermal keratinocytes in culture, a cascade of molecular events induced by injury ranging from early tyrosine kinase induction to the late upregulation of growth factors, proteases, and ECM components. Among these molecules we have found laminin-5, uPA, MMP-9, IL-1, IL-8, HB-EGF, and TGF-β that are well known healing markers stimulated, *in vivo*, during wound healing (Martin, 1997). Thus, our *in vitro* model represents a biologically relevant system to study the molecular mechanisms responsible for epidermal wound repair.

The most obvious immediate signal detected after injury of keratinocytes is tyrosine kinases activation. This phenomenon is rapid and transient and results in the phosphorylation of multiple proteins. The role of tyrosine kinases in the initiation of epithelial migration and activation has already been suggested and the presence of focal adhesion kinase and src kinase has been documented in wounds (Gates *et al*, 1994; Owens *et al*, 2000; Yamada *et al*, 2000; Kim *et al*, 2001). Immunofluorescence experiments using anti-phosphotyrosine antibodies have shown that, in healing tissues, tyrosine kinases are redistributed in migrating cells at the wound bed interface (Donaldson *et al*, 1995); however, the nature and role of tyrosine phosphorylated proteins in the healing process remain poorly documented. Here, we describe one biologic system that allows the detection of a wide number of phosphoproteins induced in healing NHK. Most of them are as yet unidentified but could be characterized in the near future using large-scale analytical techniques such as proteomics (Aulak *et al*, 2001).

Chronologically, the activation of tyrosine kinases induced by injury is followed by a strong stimulation of the three MAPK: ERK1/2, JNK, and p38[MAPK]. JNK and p38[MAPK] stimulation are transient and rapid, whereas ERK1/2 stimulation is sustained and biphasic, which is in favor of a role for this kinase in proliferation (Meloche *et al*, 1992). The activation of MAPK (p38[MAPK] and ERK) in wounded keratinocytes has been thought to occur (Jaakkola *et al*, 1998) but was never directly demonstrated until now. Indeed, in contrast with intestinal and endothelial cells (Dieckgraefe *et al*, 1997; Goke *et al*, 1998; Pintucci *et al*, 1999), the detection of p42/44 ERK, p38[MAPK], and JNK was not possible in wounded NHK cells using classical *in vitro* wounding techniques. Using an appropriate sensitive wounding system, we demonstrate that three MAPK are effectively stimulated after injury, and we also show that it is possible to identify upstream (ras activation) and downstream (c-Jun and activator transcription factor phosphorylation) events of these different pathways. The events that initiate the activation of the MAPK pathways have not yet been identified. Indeed several peptides that are activated in our system in response to injury (IL-1, TGF-β, HB-EGF, IL-8) are known to stimulate MAPK. In our system, however, the kinetics of induction of these molecules are not consistent with the immediate activation of MAPK (within minutes following injury) suggesting that MAPK activation is unlikely to be mediated through a paracrine mechanism but might be directly caused by cellular stress during wounding.

Classically, the activation of MAPK precedes the induction of early transcription factor genes that could mediate nuclear responses (Karin, 1995). The induction of *c-jun* and *c-fos* mRNA and the strong stimulation of AP-1 transcription factor activity that we observed in wounded NHK support this hypothesis. AP-1 sites are present in the promoters of several genes upregulated during wound healing, including metalloproteinases and ECM components (Angel *et al*, 2001), which is consistent with the involvement of this transcription factor in wound healing. For example, AP-1 is mandatory for the activity of the enhancer element FiRE that is selectively upregulated in keratinocytes at the

edge of the wound (Jaakkola *et al*, 1998). AP-1, however, has never been shown to be directly upregulated in healing keratinocytes; moreover, the composition of the different AP-1 complexes potentially involved in healing remains to be defined. Here we demonstrate a strong AP-1 induction in injured NHK. At least six distinct complexes involving different combinations of c-Jun and c-Fos family members (c-Fos/JunB, c-Fos/JunD, Fra-2/JunB, Fra-2/JunD, FosB/JunD, and FosB/JunB) can be identified after injury, which corroborates the idea that AP-1 may regulate the expression of several different genes during wound healing (Ma *et al*, 1997; Virolle *et al*, 1998; Corbi *et al*, 2000; Angel *et al*, 2001; Verrecchia *et al*, 2001). The nature of the healing genes driven by each specific AP-1 complex is of great interest and could be determined for example by selectively inactivating each AP-1 complex and analyzing the expression of each healing marker under these conditions.

In the near future, the objective will be to test the effect of mechanical injuries on the different classical signal transducers such as phosphoinositide 3 kinase, protein kinase C, phospholipase Cγ, and GTPase, among others. This could provide essential information for understanding the molecular mechanisms regulating the repair process in normal and pathologic conditions. The characterization of the different molecular pathways activated after injury and the comparison with data obtained from normal healing cells will be a powerful approach to characterize the molecular defects associated with the different healing syndromes.

In summary, we have characterized a series of molecular events activated *in vitro* in normal keratinocytes after injury and that likely participate in wound healing. This study has necessitated the development of a wounding device specifically designed to approach *in vitro* wound healing in keratinocytes and skin cells. The study of skin wound healing, however, cannot be limited to the identification of the molecular events involved in epidermal repair. Nevertheless, it is an established fact that *in vitro* studies are essential to improve knowledge of wound healing. This assumption is confirmed by the fact that a great number of genes activated *in vivo* are also stimulated *in vitro*. Along these lines, our biologic system, coupled with the use of specific pharmacologic inhibitors or dominant negative variants of known signal transducers, can be a powerful tool to probe the relationship between early signaling pathways and the expression of healing markers and later changes in morphology, migration, and proliferation of the cells at the leading edge of the wound. This *in vitro* system complements the classical *in vivo* models by providing the possibility of obtaining molecular data essential for understanding the normal healing process and studying the molecular anomalies involved in physiopathologic healing conditions.

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