

Co-treatment with hepatocyte growth factor and TGF- β 1 enhances migration of HaCaT cells through NADPH oxidase-dependent ROS generation

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Abbreviations: DCF-DA, 2',7'-dichlorofluorescein diacetate; NOX, NADPH oxidase; ROS, reactive oxygen species

Abstract

Wound healing requires re-epithelialization from the wound margin through keratinocyte proliferation and migration, and some growth factors are known to influence this process. In the present study, we found that the co-treatment with hepatocyte growth factor (HGF) and TGF- β 1 resulted in enhanced migration of HaCaT cells compared with either growth factor alone, and that N-acetylcysteine, an antioxidant agent, was the most effective among several inhibitors tested, suggesting the involvement of reactive oxygen species (ROS). Fluorescence-activated cell sorter analysis using 2',7'-dichlorofluorescein diacetate (DCF-DA) dye showed an early (30 min) as well as a late (24 h) increase of ROS after scratch, and the increase was more prominent with the growth factor treatment. Diphenyliodonium (DPI), a potent inhibitor of NADPH oxidase, abolished the increase of ROS at 30 min, followed by the inhibition of migration, but not the late time event. More precisely, gene knockdown by shRNA for either Nox-1 or Nox-4 isozyme of gp91phox subunit of NADPH oxidase abolished both the early time ROS production and migration. However, HaCaT cell migration was not enhanced by treatment with H₂O₂. Collectively, co-treatment with HGF and TGF- β 1 enhances keratinocyte migration, accompanied with ROS generation through NADPH oxidase, involving Nox-1 and Nox-4 isozymes.

Keywords: cell movement; hepatocyte growth factor; keratinocytes; NADPH oxidase; reactive oxygen species; transforming growth factor β 1

Introduction

Wound healing after injury is a complex process encompassing many cellular and biochemical events to restore tissue integrity. Several growth factors, synthesized mainly by activated macrophages are released from wound region during these phases in paracrine or autocrine manner (Singer and Clark, 1999). It is well known that released growth factors and cytokines control tissue repair process: Exogenous addition of growth factors such as PDGF, fibroblast growth factor (FGF), epidermal growth factor (EGF), TGF- α and TGF- β 1 to animal wounds usually accelerates tissue repair rate by enhancing granulation tissue formation or keratinocyte migration (Robinson, 1993; Steed, 1998; Carter, 2003).

Hepatocyte growth factor (HGF) is secreted mainly by mesenchymal cells such as fibroblasts and leads to motility, migration, and morphological changes in several cell types (Zarnegar and Michalopoulos, 1995). HGF also plays important roles in wound healing process, in that it primes neutrophils, facilitates migration of endothelial cells and epithelial cells, and enhances matrix synthesis and matrix deposition (Conway *et al.*, 2006). TGF- β 1 is a member of large family of growth factors involved in regulation of cellular growth and differentiation (Kingsley, 1994; Taipale *et al.*, 1998). Among many molecules known to influence wound healing, TGF- β 1 has the broadest spectrum of actions, affecting all cell types that are involved in all stages of wound healing. Both positive and negative effects of TGF- β 1 on wound healing have been reported (Wang *et al.*, 2006). Although HGF and TGF- β 1 usually acts antagonistically in many systems, the reports on the positive effect of TGF- β 1 in skin wound healing led us to assume that HGF and TGF- β 1 might not act antagonistically on keratinocyte migration, and to compare the effect of co-treatment with HGF and TGF- β 1 with either growth factor alone in keratinocyte migration.

Stimulation of the cells with growth factors leads

to the activation of diverse intracellular signaling molecules. It is also widely recognized that cells produce intracellular reactive oxygen species (ROS) when stimulated by growth factors. Production of ROS regulates signaling pathway and gene expression involving cell proliferation, survival, apoptosis or inflammation (Finkel, 1998; Gamaley and Klyubin, 1999; Finkel, 2000; Finkel and Holbrook, 2000). Regarding the source of ROS after activation by growth factor, NADPH oxidase in plasma membrane is one of the primary candidates along with xanthine oxidase, cytochrome p450, cyclooxygenase (COX), lipoxygenase (LO) existing inside the cell as well as mitochondria (Logan, 2006).

In this study, we found that the co-treatment with HGF and TGF- β 1 resulted in enhanced migration of human keratinocyte HaCaT cells, compared with either growth factor alone. Moreover, we found that the enhanced cell migration was found to be dependent on ROS generation mediated by NADPH oxidases. Our data provide potential implication of the combined use of HGF and TGF- β 1 to enhance wound healing and the understanding of the importance of NADPH-generated ROS in this process.

Results

Co-treatment with HGF and TGF- β 1 enhances HaCaT cell migration

To gain insight into the function of HGF and/or TGF- β 1 in keratinocyte migration, we performed scratch wound assay using HaCaT human keratinocytes with a micropipette tip in the presence or absence of growth factors. Whereas HGF induced narrowing of the scratch wound, TGF- β 1 showed less effect. Interestingly, we found that co-treatment with HGF and TGF- β 1 resulted in enhanced cell migration compared with either growth factor alone (Figures 1A and 1B). Since growth factors such as HGF promote cell proliferation, it might be possible that the enhanced narrowing of the wound was due to their effects on cell proliferation. However, whereas cell proliferation was increased in 10% serum condition compared to 0.5% serum condition, cell migration measured by wound healing assay was not the case. In addition, co-treatment of the wounded cells with HGF and TGF- β 1 resulted in less cell proliferation than with HGF alone (Figure 1C), suggesting that just the increase of cell proliferation could not induce the observed enhancement of wound healing. We further addressed the effect of the growth factors on cell migration by using trans-well chamber, and

found that co-treatment with both growth factors again significantly induced cell migration compared with either growth factor alone (Figure 1D), thus confirming that the increase of cell migration most probably was the cause of enhanced wound healing by co-treatment with HGF and TGF- β 1. Interestingly, while HGF induced wound healing better than TGF- β 1, TGF- β 1 induced far more trans-well migration than HGF (Figures 1B and 1D). It might be related with the difference of two assays: While the former uses attached cells, the latter uses single cells.

ROS generation is necessary for the enhancement of cell migration by combined growth factor treatment

To verify the signal transduction pathway responsible for the increase of re-epithelialization, well-known inhibitors of various signaling pathways were pre-treated before the wound generation and cytokine treatment. Among the inhibitors tested, N-acetylcysteine (NAC), an antioxidant, along with PD98059 were the most effective ones in inhibiting re-epithelialization (Supplemental Data Figure S1), suggesting the involvement of ROS and ERK signaling in this process. Since ROS is widely recognized as one of the diverse intracellular signaling molecules when stimulated by growth factors, we next performed ROS analysis by using FACS and confocal microscope after staining with DCF-DA dye. FACS analysis revealed that ROS level slightly but statistically significantly increased after the scratch at both early (30 min) and late (20 h) time points when treated with both HGF and TGF- β 1 at both 30 min and at 24 h compared with those without growth factor treatment (Figure 2A). Observation of DCF-DA fluorescence by confocal microscopy also revealed the increase of ROS by growth factor treatment. Notably, the increase of ROS was evident mainly at the margins of wound, suggesting that the scratch-induced damage to the cells was the source of initial signaling for ROS generation (Figure 3B, upper panels). To address whether the ROS generation is essential in HaCaT cell migration, N-acetylcysteine, an antioxidant, was treated prior to the application of growth factors and wound generation. Indeed, N-acetylcysteine significantly abolished cell migration in a dose-dependent manner (Figure 2B), suggesting the strong involvement of ROS in this process.

Diphenyliodonium significantly inhibits the increase of ROS at early time point, but not at late time point, followed by inhibition of cell migration

Originally, ROS were recognized as an instrument

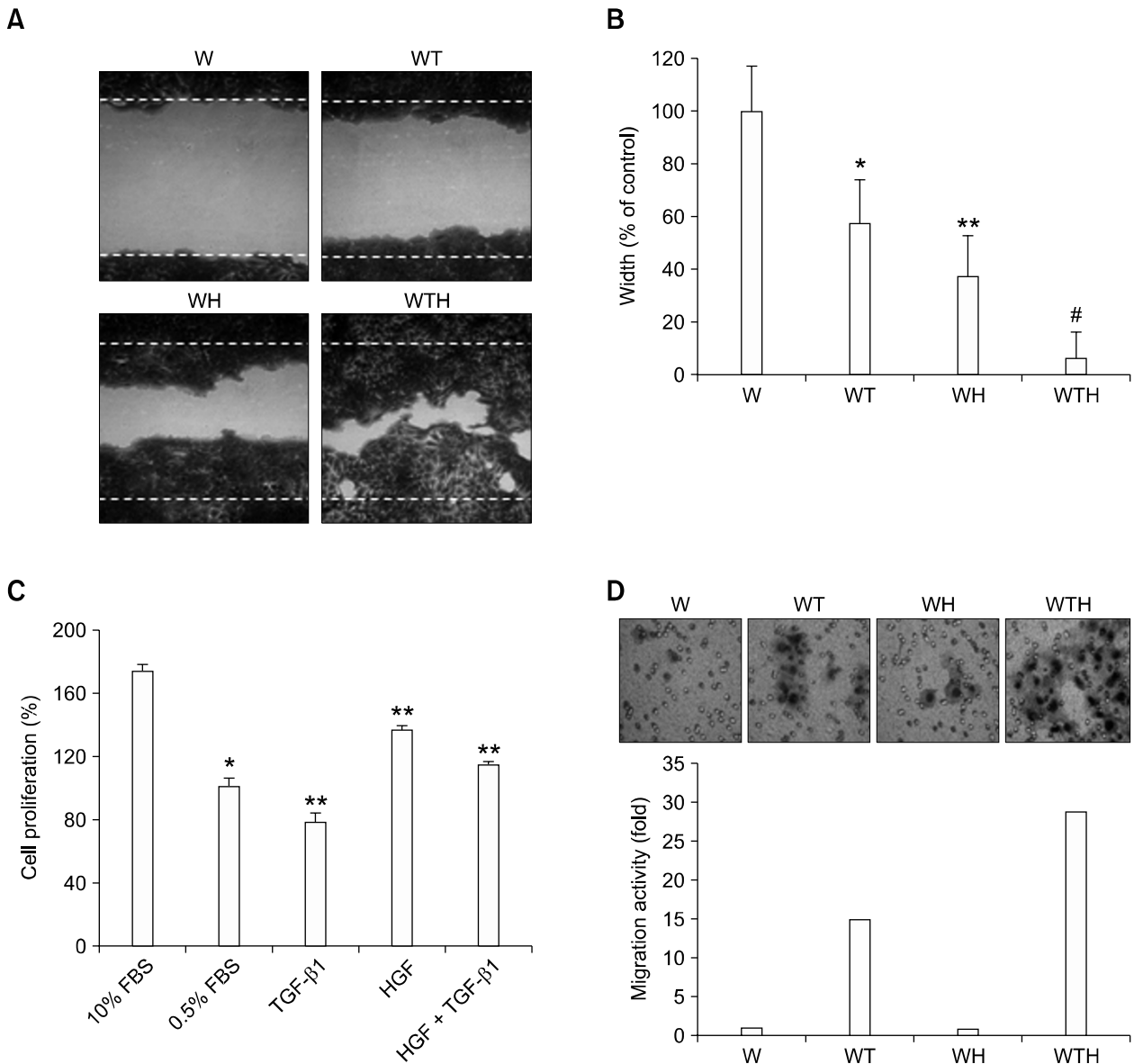


Figure 1. Co-treatment with HGF and TGF-β1 enhanced HaCaT cell migration. (A) Scratch wound assay. HaCaT cells were seeded in 24-well plates. After 24 h, cells were scratched using a micropipette tip, and growth factors were added as indicated. After incubation for 24 h, the indicated wells were stained with crystal violet. Representative pictures from three independent experiments are shown. (B) The width of the wound was measured by using ocular lens with ruler. Data are presented as mean ± SD of three independent experiments. W: No treatment, WT: TGF-β1 2 ng/ml, WH: HGF 30 units/ml, WTH: HGF 30 units/ml + TGF-β1 2 ng/ml ($n = 25$, * and **, $P < 0.01$ compared to control, #, $P < 0.01$ between WH and WTH; student's t -test). (C) Cell proliferation assay. HaCaT cells were seeded in 96-well plates before the respective growth factors were added. After 24 h of the treatment with growth factors as indicated, MTT assay was performed. ($n = 6$, *: $P < 0.01$ compared to 10% FBS group, **: $P < 0.01$ compared to 0.5% FBS group by student's t -test). Data are presented as mean ± SD of two independent experiments. (D) Cell migration assay. HaCaT cells were seeded at a density of 1.0×10^4 cells/well in upper chamber prior to the treatment with cytokines indicated. After 24 h of the treatment with cytokines, cells were fixed with acetone: methanol (1:1), and stained with crystal violet. Representative pictures from two independent experiments are shown.

for mammalian host defense, and early works led to the characterization of the respiratory burst of neutrophils and finally the NADPH oxidase complex, which is now recognized as a primary source of ROS. To address whether the origin of ROS production in our system was NADPH oxidase

complex, we pre-treated HaCaT cells with diphenyliodonium (diphenyliodonium; 5 μM), an NADPH oxidase inhibitor, before growth factor treatment that was followed by scratch. Interestingly, pre-treatment of the cells with diphenyliodonium resulted in a complete inhibition of early time point

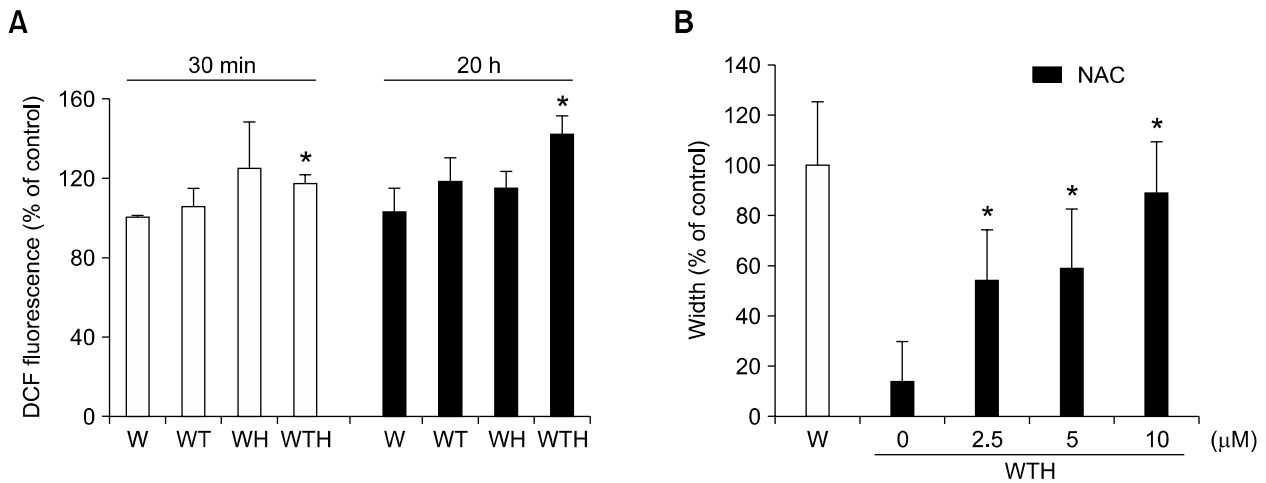


Figure 2. ROS induction at early and late time points following scratch wound was enhanced by combined growth factor treatment. (A) Enhanced ROS production by co-treatment with HGF and TGF- β 1. HaCaT cells were scratched using a comb and cytokines were treated as indicated. After incubating for 30 min or 20 h, cells were incubated for 10 min with DCF-DA, and then harvested for the FACS analysis. Data are presented as mean \pm SD of three independent experiments (*; $P < 0.01$ compared to that without cytokine treatment by student's t -test). (B) N-acetylcysteine (NAC) could block the re-epithelialization of HaCaT cells induced by co-treatment with HGF and TGF- β 1. HaCaT cells were pre-treated with or without indicated concentrations of NAC (2.5 μ M, 5 μ M, 10 μ M) for 30 min before scratched and growth factors were added as indicated. Data are presented as mean \pm SD of three independent experiments.

ROS production in response to scratch wound itself as well as to the combined growth factor stimulation (Figure 3A, left, arrowheads). On the other hand, ROS generation at 20 h was not abolished by diphenyliodonium treatment (Figure 3A, right), which was the case even with re-application of diphenyliodonium at 18 h post-scratch (data not shown), indicating that the failure of diphenyliodonium to inhibit the late-time point ROS generation was not due to possible instability of the inhibitor in culture medium. Observation of DCF fluorescence by confocal microscopy confirmed the inhibition of ROS generation by diphenyliodonium in response to scratch wound itself as well as to the combined growth factor stimulation (Figure 3B, lower panels).

We then asked whether diphenyliodonium treatment of the cells could inhibit HaCaT cell migration. Indeed, diphenyliodonium partially, but significantly abolished wound healing in a concentration-dependent manner (Figure 3C). Therefore, ROS generation by NADPH oxidase seemed to be necessary for the migration of HaCaT keratinocytes after the treatment with HGF and TGF- β 1.

Knockdown of either Nox-1 or Nox-4 isozyme of p91^{phox} subunit of NADPH oxidase inhibits ROS production and HaCaT cell migration

We next tried to further confirm the involvement of NADPH oxidase complex by using molecular bio-

logical approach. NADPH oxidase complex consists of various subunit proteins, including p91^{phox} isozymes. Since Nox-1 and Nox-4 were reported to be present in HaCaT cells (Chamulitrat *et al.*, 2004), we tested whether knock-down of Nox-1 or Nox-4 using specific shRNAs in HaCaT cells could abolish the ROS generation. Indeed, transfection of either shRNA for Nox-1 or Nox-4, but not control shRNA, almost completely abolished ROS production in response to scratch wound itself as well as to the combined growth factor stimulation (Figure 4A), confirming the involvement of NADPH oxidase complex in wound-induced as well as growth factor-induced ROS generation.

In line with the inhibition of HaCaT migration by diphenyliodonium, we next addressed whether Nox-1 or Nox-4 depletion also inhibited the wound healing. As shown in Figure 4B, knock-down of either Nox-1 or Nox-4 effectively inhibited the wound healing by co-treatment with HGF and TGF- β 1. These results demonstrated that NADPH oxidase-mediated ROS production is essential for promotion of HaCaT cell migration, and both Nox-1 and Nox-4 are necessary in this process.

PI3K pathway is involved in re-epithelialization process, but not in ROS generation

Non-phagocytic cells produce superoxide anions in response to growth factors including PDGF and EGF (Sundaresan *et al.*, 1995; Bae *et al.*, 1997).

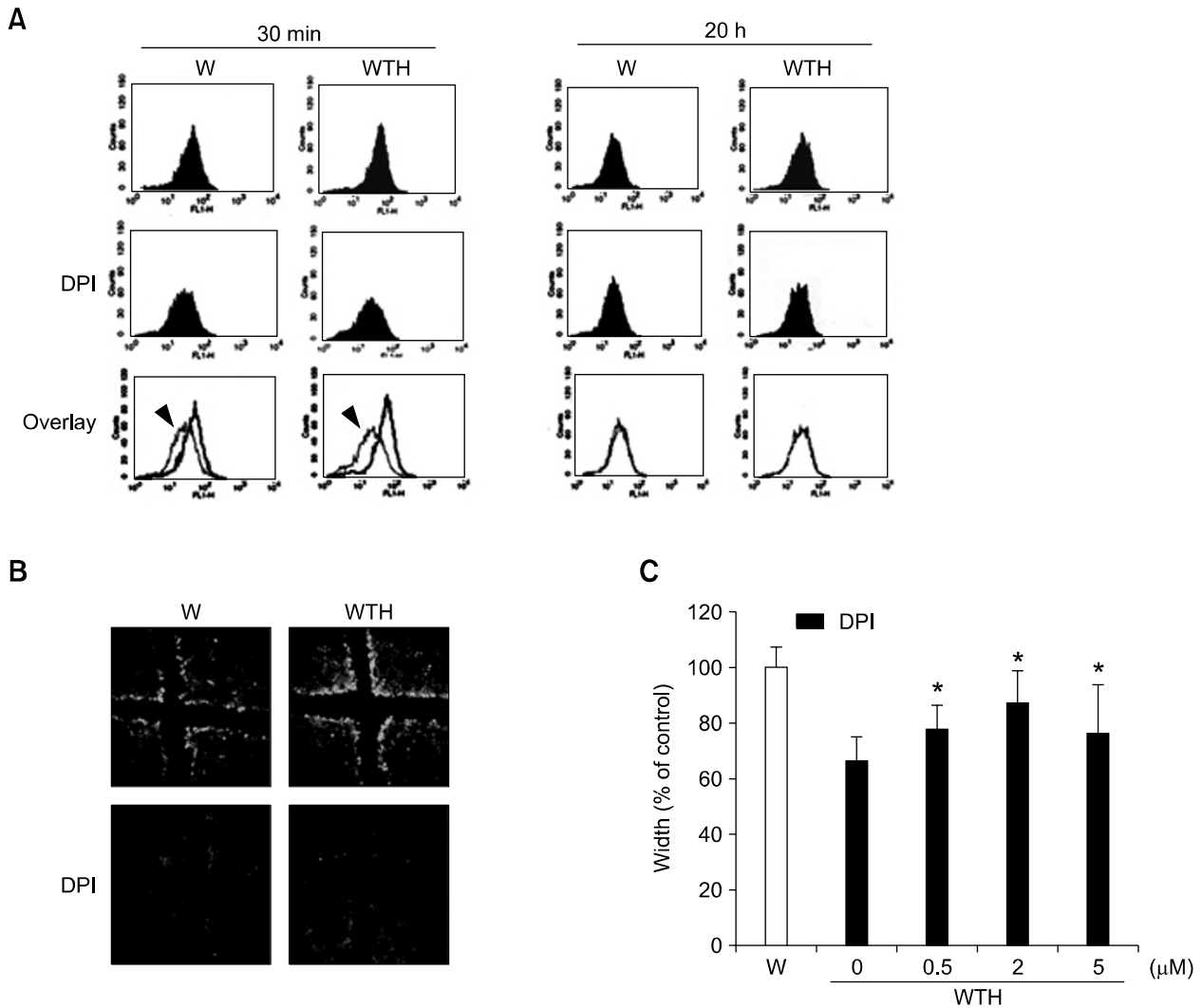


Figure 3. Diphenyliodonium (DPI) inhibited the increase of ROS significantly at early time point, but not at late time point, followed by inhibition of HaCaT cell migration. (A) and (B) diphenyliodonium inhibited significantly the increase of ROS at early time point. (A) HaCaT cells were pretreated with diphenyliodonium (5 μM) for 1 h before scratching, and growth factors for 30 min were added as indicated. Representative images of FACS analysis of three independent experiments are presented. Arrowheads denote FACS profiles of diphenyliodonium-treated samples. (B) Diphenyliodonium as well as growth factors were applied as described in (A). After incubation with DCF-DA, cells were observed under a confocal microscope. Representative images from two independent experiments are presented. (C) Migration of HaCaT cells was inhibited by diphenyliodonium treatment. HaCaT cells were pretreated for 1 h with indicated concentrations of diphenyliodonium before scratching and growth factors were applied as indicated. Data are presented as mean ± SD of two independent experiments (*; $P < 0.05$ compared to that without diphenyliodonium by student's *t*-test).

Especially, ROS production in PDGF-stimulated cells has been shown to be mediated by sequential activation of phosphatidylinositol 3- kinase (PI3K), βPix, and Rac1, which then binds to Nox1 to stimulate its NADPH oxidase activity (Bae *et al.*, 2000; Park *et al.*, 2004). Therefore, we next investigated the involvement of PI3K pathway in the ROS generation and wound healing process. Interestingly, whereas HaCaT cell migration was inhibited in dose-dependent manners by either wortmannin or LY294002 (Figures 5A and 5B), wortmannin or LY294002 did not abolish the

increase of ROS at both early (30 min; Figures 5C and 5D) and late time points (20 h; data not shown). These results suggest that PI3K pathway is indeed involved in HaCaT keratinocyte migration by growth factor co-treatment, but not through affecting ROS production. In fact, when we treated various signaling inhibitors prior to the addition of growth factors and wound generation, we observed that several different inhibitors could abolish the HaCaT cell migration (Supplemental Data Figure S1), which suggests that keratinocyte migration is a rather complex process which requires the har-

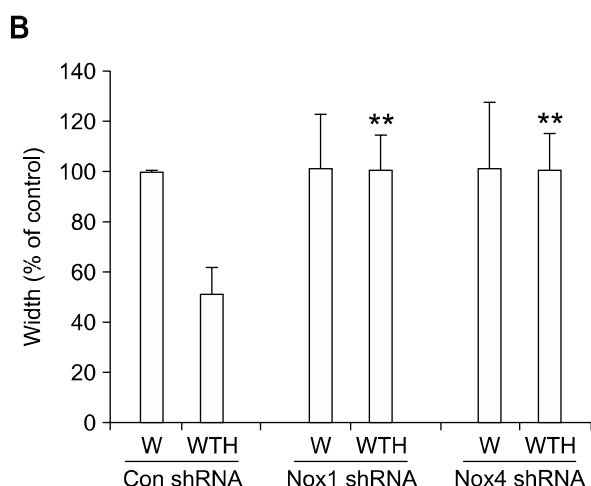
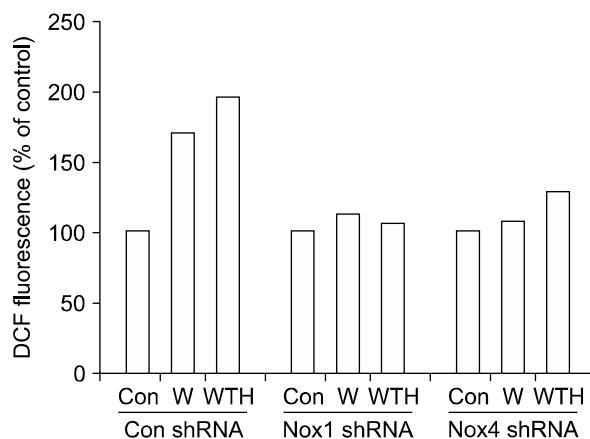
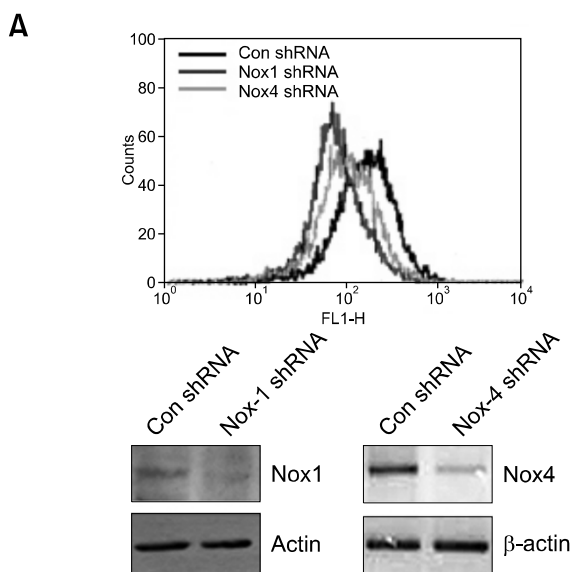


Figure 4. Knock-down of Nox-1 or Nox-4 confirmed the involvement of NADPH oxidase in ROS generation after wound. (A) Depletion of either Nox-1 or Nox-4 significantly inhibited ROS production. After indicated plasmids were electroporated cells were scratched and growth factors were added as indicated. Thirty min later, cells were harvested and subjected to FACS analysis after DCF-DA staining. Representative data from two independent experiments are shown (right panel), and the extent of knock-down of Nox-1 and Nox-4 was measured by western blotting and RT-PCR, respectively (left lower panel). Representative FACS profiles are overlaid to reveal the differences between samples. Only the differences between samples with both wound and growth factor treatment were shown (left upper panel). Con shRNA: pSuper plasmid itself transfected cells. (B) HaCaT cell migration was inhibited by depletion of either Nox-1 or Nox-4. Cells harboring indicated shRNAs were subjected to scratch wound assay. Data are presented as mean \pm SD of two independent experiments (**; $P < 0.01$ compared to pSuper-transfected cells by student's t-test).

monious involvement of various intracellular signaling systems.

H₂O₂ is not sufficient for inducing HaCaT cell migration

We found that ROS production by co-treatment of HaCaT cells with HGF and TGF- β 1 was necessary for wound migration (Figure 3). Therefore, to address whether ROS generation is sufficient for migration, scratch wound assay was performed in the presence of different concentrations of H₂O₂. As shown in Figure 6A, HaCaT cell migration was not enhanced by H₂O₂ within a concentration range tested, indicating that the increase of ROS production such as H₂O₂ is necessary but not sufficient for migration. However, there is a possibility that the cytotoxic effect of H₂O₂ at high concen-

tration range (Figure 6B) would interfere with the possible wound healing effect of H₂O₂ at these concentration ranges.

Discussion

ROS, such as superoxide anions and hydrogen peroxide (H₂O₂), is produced in mammalian cells in response to the activation of various cell surface receptors, and contribute to intracellular signaling and regulation of various biological activities (Finkel, 2000; Lambeth *et al.*, 2000). Receptor-mediated ROS production has been extensively studied in phagocytic cells. The enzyme NADPH oxidase of such cells is composed of at least five protein components, namely two transmembrane flavocytochrome b components

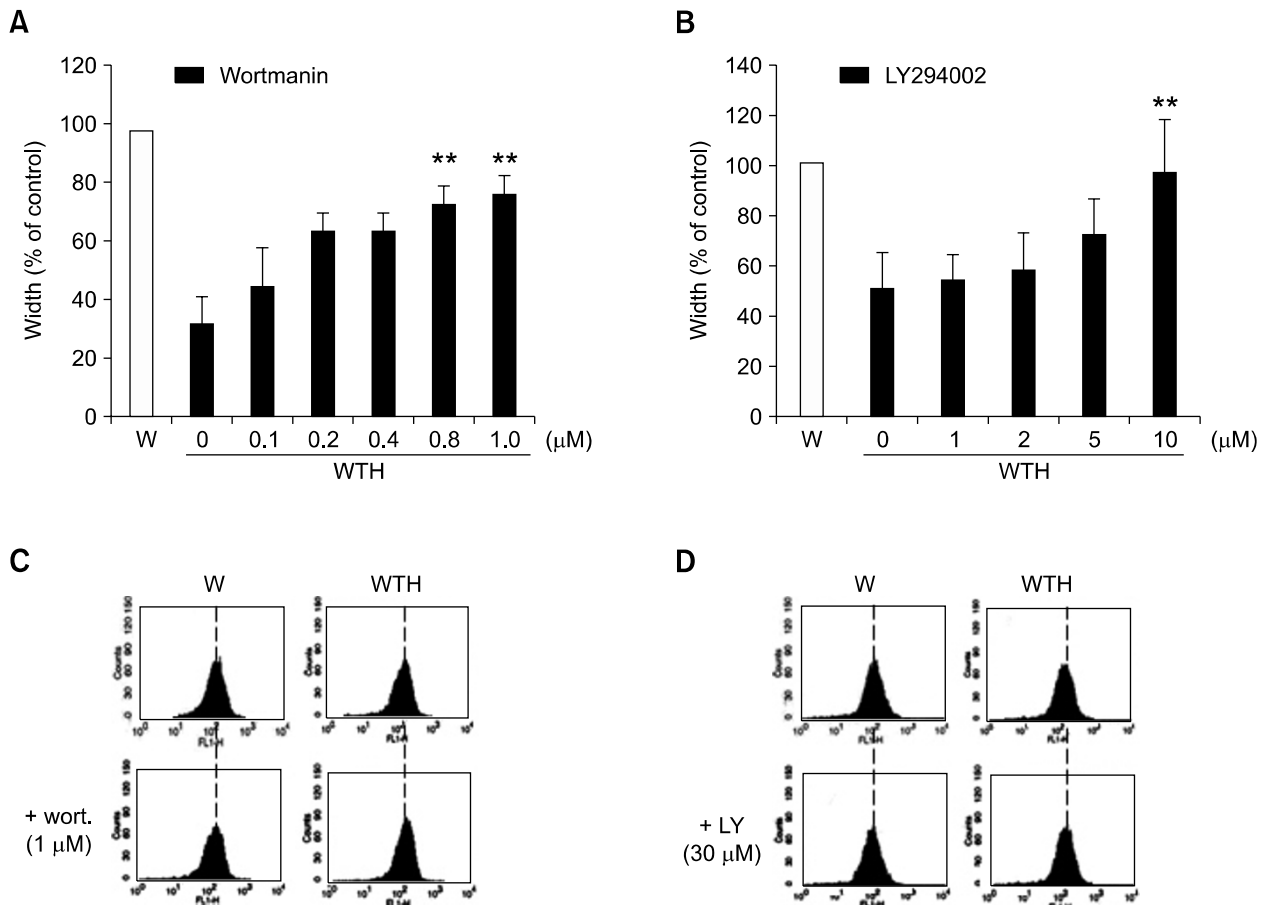


Figure 5. PI3K pathway was involved in cell migration, but not in ROS generation. (A) and (B) HaCaT cell migration was inhibited by PI3K inhibitors. HaCaT cells were pretreated for 1 h with wortmannin (A) or LY294002 (B) at the indicated concentrations before scratching. Growth factors were added as indicated just after the scratch. Data are presented as mean \pm SD of two independent experiments. (**; $P < 0.01$ compared to that without inhibitors by student's *t*-test). (C) and (D) Wortmannin (1 μ M) and LY294002 (30 μ M) could not abolish the increase of ROS. HaCaT cells were prepared as indicated (A). Then, cells were incubated for 10 min with DCF-DA harvested for FACS analysis. Peak fluorescence intensity was measured for each sample, and a representative data from two independent experiments are shown. Vertical dashed line was drawn to help compare the location of two peaks.

(gp91^{phox} and p22^{phox}) and three cytosolic components (p47^{phox}, p67^{phox}, and p40^{phox}) (Babior, 1999). The exposure of resting phagocytic cells to an appropriate stimulus results in extensive phosphorylation of the cytosolic components of NADPH-oxidase and their association with the transmembrane flavocytochrome b components (Ago *et al.*, 1999; Cross *et al.*, 1999). The assembled oxidase complex catalyzes the transfer of an electron to O₂ to yield superoxide anion and H₂O₂. Several homologs (Nox-1, Nox-3, Nox-4, Nox-5, Duox1, and Duox2) of gp91^{phox} (Nox-2) have been identified in various non-phagocytic cells (Cheng *et al.*, 2001). Based on the effect of Nox-1 antisense RNA in smooth muscle cells, Nox-1 has been implicated in PDGF-induced ROS production (Lassegue *et al.*, 2001). Nox-4 has also been reported to play a central role in LPS-induced pro-inflammatory responses of endothelial cells in

an ROS-dependent manner (Park *et al.*, 2006), and is shown to be expressed in melanoma cells as well as normal human epidermal melanocytes (Brar *et al.*, 2002). To the best of our knowledge, this is the first report showing the involvement of Nox-1 and Nox-4 in keratinocyte migration.

Although ROS generation was focused as one of the responsible signaling pathways in keratinocyte migration, it seems that numerous different major signaling pathways are necessary for the completion of this process: (i) Exogenous addition of H₂O₂ was not sufficient to induce cell migration (Figure 6), (ii) the inhibition of PI3K with two different inhibitors also prevented HaCaT cell migration without affecting ROS level (Figure 5), (iii) several different inhibitors could abolish the HaCaT cell migration (Supplemental Data Figure S1). These data strongly argues that several different downstream signaling pathways are in-

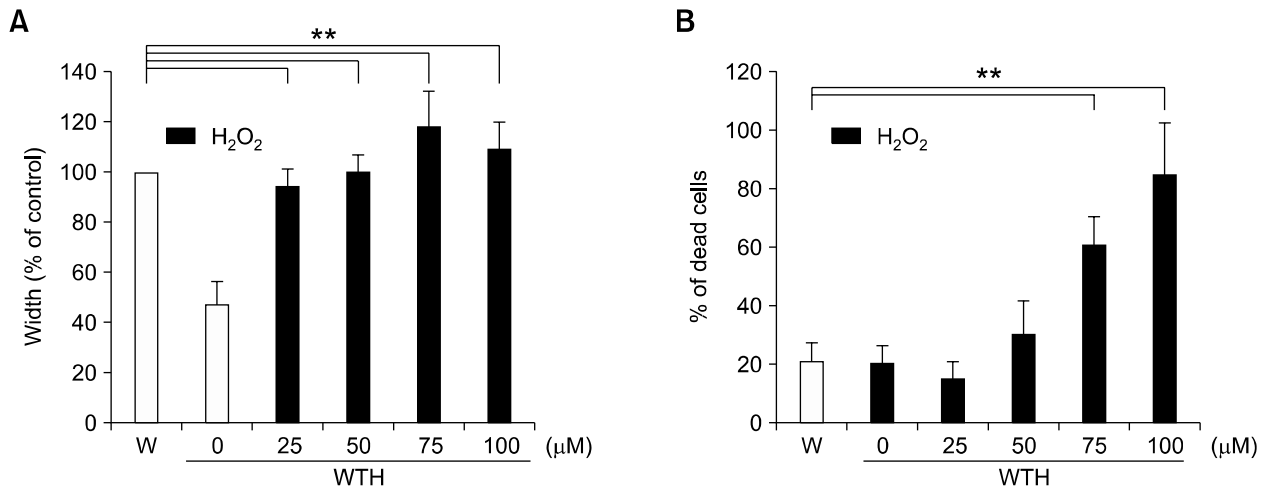


Figure 6. HaCaT cell migration was not enhanced by H₂O₂ treatment. HaCaT cells were scratched and different concentrations of H₂O₂ were added as indicated. After incubation for 24 h, the respective wells were stained with crystal violet to measure the wound closure (A) or cells were harvested and stained by trypan blue to measure the cell death (B). Data are presented as mean \pm SD of two independent experiments (**: $P < 0.01$ compared to that with cytokine treatment by Student's *t*-test).

involved in keratinocyte migration.

Another well-documented mechanism of enhanced keratinocyte migration other than ROS generation is the secretion of EGF family ligands such as EGF, TGF- α , amphiregulin, and HB-EGF, which leads to the autocrine activation of EGFR (Coffey *et al.*, 1987; Cook *et al.*, 1991; Higashiyama *et al.*, 1991). Interestingly, Ellis *et al.* (2001) showed that scrape-wounding epithelial cell monolayers induce HB-EGF mRNA expression by a mechanism that most probably require Erk-1 and -2 activation, leading to the migration of the cells via activation of EGFR (Ellis *et al.*, 2001). Notably, phosphorylation of both Erk-1 and -2 was significantly increased and sustained by treatment with a combination of both growth factors in our system, and signal inhibitors such as PD98059 and U0126 showed dose-dependent inhibition of HaCaT cell migration without any significant effect on cell proliferation (unpublished observation by Nam). However, we could not observe the activation of EGFR in our system (Supplemental Data Figure S3).

Re-epithelialization by proliferation and migration of keratinocytes from the margin is one of the principal steps in the complex process of wound healing. Thus, enhancement of re-epithelialization would be beneficial in wound healing process. We have shown in the present study that co-treatment of wounded HaCaT cells with HGF and TGF- β 1 enhanced keratinocyte migration. Since the use of growth factors as a therapeutic measure to enhance wound healing process appears to be a promising approach, it would be very interesting to test whether combined growth factor treatment

would also be beneficial for wound healing *in vivo*.

Methods

Cell culture

The spontaneously immortalized human keratinocyte cell line HaCaT cells (a kind gift from Dr. HY Kang at the Department of Dermatology, Ajou University School of Medicine, Suwon, Korea) were grown in RPMI-1640 medium (Gibco Life Technologies, Rockville, MD) supplemented with 10% (v/v) heat-inactivated FBS (Gibco) at 5% CO₂ in a humidified atmosphere at 37°C.

Chemicals

Recombinant human hepatocyte growth factor (rhHGF) was generously provided by Dr. G. VandeWoude (Laboratory of Molecular Oncology, Van Andel Research Institute, Grand Rapids, MI). HGF concentrations are presented as scatter units/ml; and 5 units are equivalent to approximately 1 ng of the protein. Recombinant TGF- β 1 was purchased from R&D systems (Minneapolis, MN). 2', 7'-dichlorofluorescein diacetate (DCF-DA) was purchased from Molecular Probe Corp. (Eugene, OR). Diphenylidodinium and N-acetylcysteine were from Sigma-Aldrich (St Louis, MO). FBS, trypsin-EDTA, and antibiotic-antimycotic containing penicillin G sodium were obtained from Gibco BRL. Protease inhibitor cocktail tablet was purchased from Roche Applied Science (Mannheim, Germany). Phosphatase inhibitors and other reagents, unless specified, were from Sigma Chemical Co.

Scratch wound motility assay

HaCaT cells were seeded (3×10^5 cells/well) into 24-well

plates in RPMI-1640 supplemented with 10% FBS and allowed to adhere overnight. Cells were then starved with 0.5% FBS containing media. The mono-layers were then carefully scratched with sterile blue pipette tips and incubated with 0.5% serum-containing medium with HGF (30 units/ml) and/or TGF- β 1 (2 ng/ml). After 36 h, cells were washed three times with cold PBS, and then fixed in acetone:methanol (1:1, v/v) for 15 min on ice and stained with 2% (w/v) crystal violet. The width of the wound was measured by using ocular lens with ruler.

Cell proliferation assay

To examine the effect of HGF (30 unit/ml) and TGF- β . (2 ng/ml) on cell proliferation, MTT assay was performed with Cell Proliferation Kit I [MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] by following the manufacturer's instruction (Roche Applied Science, Germany).

Migration assay

HaCaT cells were seeded (1×10^4 cells/well) into 24-well plates in RPMI-1640 containing 0.1% FBS onto the micro-porous membrane (8.0 μ m) in the upper chamber of the Transwell[®] (Corning, NY). Twenty-Four h after the treatment with cytokines, the unmigrated cells in the upper chamber were gently removed by using a cotton swab. Cells which had migrated through the membrane to the lower chamber were fixed, stained with crystal violet and washed. Migration was determined by counting the cells on the lower surface of the filter by phase-contrast microscopy at $\times 100$ magnification.

Inhibitor study

Wounded HaCaT cells were pre-treated for 30 min with various inhibitors of cell signaling. After then, both HGF (30 units/ml) and TGF- β 1 (2 ng/ml) were added to the cells for 30 h along with indicated inhibitors. After staining with crystal violet, width of the wound was measured by using ocular lens with ruler.

Intracellular H₂O₂ concentration

HaCaT cells were seeded in 60 mm-petri dishes. After 24 h, culture media were changed with fresh RPMI-1640 media containing 0.5% FBS. After another 24 h, confluent cells were scratched using a comb and cytokines were added as indicated. After incubating for 30 min or 20 h, cells were washed with PBS and incubated for 10 min in the dark at 37°C with the same solution containing 8 μ M 2',7'-dichlorofluorescein diacetate (DCF-DA; Molecular Probes). DCF-DA is oxidized by H₂O₂ to highly fluorescent 2',7'-dichlorofluorescein (DCF). The cells were then examined with a laser scanning confocal microscope (model Fluoview 300; Olympus) equipped with an argon laser tuned to an excitation wavelength of 488 nm, and a Olympus $\times 100$ objective lens. For determination of intracellular ROS level by FACS, we prepared cells in the same manner. Stained cells were washed, re-suspended in PBS, and analyzed by flow cytometry (FACS Vantage,

Becton Dickinson Corp.).

Transfection of shRNAs for Nox-1 and Nox-4

ShRNA constructs specific for Nox-1 or Nox-4 were generous gifts from Dr. YS Bae (Ewha Womans University, Seoul, Korea), and were generated as previously described (Park *et al.*, 2004). Briefly, specific sequences of 19 nucleotides of human Nox-1 (CCAGGATTGAAGTGGATGG, residues 1130 to 1148) and Nox-4 cDNA (GTCAAC-ATCCAGCTGTACC, residues 1474 to 1492) were used for the synthesis of small interfering RNAs (siRNAs). The pSUPER vector for shRNAs was purchased from Oligoengine. The phosphorylated oligonucleotides were annealed and cloned into the pSUPER vector by the use of *Bgl*III (5' end) and *Hind*III (3' end). Cells were transfected by electroporation.

Electroporation of HaCaT cells

Electroporation (Amaxa Biosystems, Cologne, Germany) was performed as described previously (Lenz *et al.*, 2003). HaCaT cells (3×10^6) were centrifugated, and the pellet was then resuspended in RPMI-1640 media containing 10% FBS. Plasmid DNAs (7 μ g) were mixed with 250 μ l of cell suspension, transferred into 4.0 mm-wide ELECTROPORATION CUVETTES PLUS[™] (A Division of Genetronics, USA), and electroporated with an Amaxa Nucleofector apparatus, using an appropriate program supplied by the manufacturer. After electroporation, the cells were immediately transferred to complete medium and cultured in 60-mm culture dishes at 37°C until analysis.

Supplemental data

Supplemental Data include three figures and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-42-4-03.pdf.

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