Effects of Angiotensin II Receptor Signaling during Skin Wound Healing

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The tissue angiotensin (Ang) system, which acts independently of the circulating renin Ang system, is supposed to play an important role in tissue repair in the heart and kidney. In the skin, the role of the system for wound healing has remained to be ascertained. Our study demonstrated that oral administration of selective AngII type-1 receptor (AT₁) blocker suppressed keratinocyte re-epithelization and angiogenesis during skin wound healing in rats. Immunoprecipitation and Western blot analysis indicated the existence of AT₁ and AngII type-2 receptor (AT₂) in cultured keratinocytes and myofibroblasts. In a bromodeoxyuridine incorporation study, induction of AT₁ signaling enhanced the incorporation into keratinocytes and myofibroblasts. Wound healing migration assays revealed that induction of AT₁ signaling accelerated keratinocyte re-epithelization and myofibroblasts recovering. In these experiments, induction of AT₂ signaling acted vice versa. Taken together, our study suggests that skin wound healing is regulated by balance of opposing signals between AT₁ and AT₂. (Am J Pathol 2004, 165:1653–1662)

Angiotensin (Ang) II is the active biological octapeptide of the systemic renin Ang system, which is a circulating humoral system responsible for blood pressure regulation and salt-water homeostasis. AngII exerts its effects through binding the specific receptors. There are two major subtypes of AngII receptors: type 1 (AT₁) and type 2 (AT₂).¹ Most of the physiological actions of AngII are regulated through AT₁. Recently, in addition to the classical role, AngII has attracted attention for its novel role as a growth factor. Growth-modulating effects of these receptors were reported in cardiac vascular endothelial cells, smooth muscle cells, and fibroblasts, where AT₁ caused cell proliferation and synthesis of extracellular matrix proteins, whereas AT₂ acted in an anti-proliferative

manner.^{2–5} Despite the fact that the role of AngII and its receptors has been well discussed in the cardiovascular field, a few reports can be found in the dermatological field. Literature has shown that the topical administration of AngII accelerated skin wound healing,^{6–8} an increased level of AngII or Ang-converting enzyme in the rat skin^{1,9} during wound healing, and the existence of AngII receptors by harvesting whole wounded skin.^{10–13} However, the mechanism of AngII on skin wound healing has remained unknown. In this report, we studied the mechanism of AngII receptor signaling using selective AT₁ or AT₂ blockers to assess the effects of AngII receptor signaling during skin wound healing.

Materials and Methods

Agents, Animals, and Wound Healing Experiments in Vivo

Candesartan (CV-11974) and Candesartan cilexetil (TCV-116) were kind gifts from Takeda Chemical Industries (Osaka, Japan). Candesartan is a potent and selective AT₁ blocker that binds tightly to and dissociates slowly from AT₁. Candesartan cilexetil is a prodrug, and is administered orally and converted rapidly and completely to its active form, candesartan, by the enzyme esterase at the time of intestinal absorption.¹⁴ PD-123319 ditrifluoroacetate (Sigma Japan, Tokyo, Japan) is a selective AT₂ blocker and used in experiments *in vitro*.^{2,3} Sprague-Dawley male rats (250 to 300 g, ~10 weeks of age) were purchased from SLC Inc., Japan and housed on a 12:12-hour light:dark cycle. Food and water were available *ad libitum*. The experiments were performed according to the institutional guidelines for care and use of laboratory animals.

TCV-116, 1 mg/kg/day ($n = 5$) or 10 mg/kg/day ($n = 5$), suspended with gum arabic in a volume of 2 ml/kg water or vehicle ($n = 5$) were orally administered to the rats once a day starting day 0 until the end of the exper-

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iment. On day 0, a full-thickness skin wound was made on the dorsal site of each rat using 8-mm biopsy trepan (Kai Industries, Seki, Japan). The wounds had 50 μ l of 70% ethanol once a day starting day 0 until day 5 after injury without bandaging. No wounds had infections. The wounds were totally excised at each selected time point (day 3, 6, 9, 12, and 15 after wounding), and were fixed in 10% formalin, embedded in paraffin, and offered for hematoxylin and eosin (H&E) or immunohistochemical staining. Rats were anesthetized with pentobarbital sodium (50 mg/kg i.p.) during wounding and tissue collection.

Analysis of Re-Epithelization in Vivo

Using the H&E sections, the extent of re-epithelization of wounds was evaluated by measuring the epidermal migration from the normal wound margin to the point where the migrating epithelium stopped processing.⁶ The re-epithelization index was determined by the percentage of the new epithelium present in the total wound.

Analysis of Vascular Growth in Vivo

To detect the blood vessels, immunohistochemical staining using the rabbit anti-von Willebrand factor polyclonal antibody (DAKO Japan, Kyoto, Japan) was performed on the sections by the labeled streptavidin-biotin method.¹⁵ The reaction was visualized using 3-amino-9-ethylcarbazole (DAKO Japan) as chromogen and the slides were counterstained with hematoxylin and mounted in aqueous mounting medium for examination. The average number of blood vessels with a diameter of more than 4 μ m per square millimeter in the wound site was calculated as vascular density. Two of the authors independently counted the number of blood vessels without any information on each section.

Cell Culture

Keratinocytes were cultured as previously described.¹⁶ In brief, epidermal strips were isolated from the surgically removed rat dorsal skin using dispase (1.2 U/ml, Sigma Japan, Tokyo, Japan) for 24 hours at 4°C. After incubation of epidermal strips in 0.05% trypsin/0.02% ethylenediaminetetraacetic acid for 25 minutes at room temperature, keratinocytes were plated on culture dishes (78.5 cm²; Falcon Plastics, Becton-Dickinson Labware, Franklin Lakes, NJ) at $\sim 4.0 \times 10^4$ cells/cm² in Defined Keratinocyte Serum-Free Medium (Life Technologies, Inc., Grand Island, NY) with the addition of the growth supplement included with the medium. Calcium level, as stated by the manufacturer, was lower than 0.1 mmol/L. The cells were grown as monolayers at 37°C in a humidified incubator with 5% CO₂/95% air.

Myofibroblasts from rat cutaneous granulation tissue were obtained as previously described.¹⁷ The cells within 17 passages were used because myofibroblasts were maintained in culture for more than 17 passages. Myofibroblasts were grown to confluence and subcultured on culture dishes (78.5 cm², Falcon Plastics) at 37°C in a

humidified incubator with 5% CO₂/95% air in Dulbecco's modified medium with 10% fetal bovine serum (Life Technologies, Inc.), penicillin (100 U/ml), streptomycin (50 μ g/ml), kanamycin (50 μ g/ml), and hydrocortisone (0.4 μ g/ml).

Immunoprecipitation and Western Blot Analyses

The epidermal strips as a source of keratinocytes, cultured keratinocytes, and myofibroblasts were washed twice with ice-cold phosphate-buffered saline, and harvested and lysed in lysis buffer containing 10 mmol/L Tris-HCl, 10 mmol/L ethylenediaminetetraacetic acid, 0.4 mmol/L phenylmethyl sulfonyl fluoride, 1 μ g/ml leupeptin, and 1% Triton X-100. After they were incubated for 1 hour at 4°C, the cell lysates were centrifuged at 12,000 $\times g$ for 10 minutes, and the supernatant was collected.¹⁸ Proteins were precleared with protein G agarose for 30 minutes at 4°C. Then anti-AT₁ (affinity-purified rabbit polyclonal anti-AT₁; Santa Cruz Biotechnology, Santa Cruz, CA) or AT₂ antibodies (affinity-purified goat polyclonal anti-AT₂, Santa Cruz) were added to the precleared samples and incubated for 1 hour at 4°C. Immune complexes were boiled in sodium dodecyl sulfate-sample buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis,¹⁹ transferred to polyvinylidene difluoride membrane,²⁰ and immunoblotted with anti-AT₁ or AT₂ antibodies, respectively. After incubation with secondary antibodies, immunoreactive proteins were detected by the enhanced chemiluminescence reaction (Amersham Bioscience Japan, Tokyo, Japan). Staining of standard proteins on the polyvinylidene difluoride membranes was performed with 0.04% Coomassie brilliant blue R-250.

Bromodeoxyuridine (BrdU) Labeling and Immunohistochemical Staining

Keratinocytes and myofibroblasts were plated on culture disks (0.79 cm²; Sumitomo Chemical, Tokyo, Japan) at $\sim 1.5 \times 10^2$ cells/cm². Subsequently, keratinocytes and myofibroblasts were exposed to AngII (100 pg/ml; Wako, Tokyo, Japan), CV-11974 (10^{-7} mol/L), CV-11974 (10^{-7} mol/L with AngII 100 pg/ml), PD-123319 (10^{-5} mol/L, dissolved in 0.9% saline), PD-123319 (10^{-5} mol/L with AngII 100 pg/ml), or vehicle (0.9% saline with 0.1 N Na₂CO₃), respectively, with BrdU (15 μ g/ml) in the medium for 24 hours and fixed in 100% ethanol. The BrdU immunohistochemistry was performed using a cell proliferation kit (Amersham Bioscience Japan). After quenching endogenous peroxidase with 3% H₂O₂, the DNA in cells was subsequently denatured with denaturing solution for 30 minutes, followed by a 1-hour incubation with biotinylated mouse monoclonal anti-BrdU antibody at 37°C. Disks were incubated with streptavidin-peroxidase for 10 minutes at room temperature. The incorporation of BrdU into the genomic DNA of proliferating cells was detected by staining with 3-amino-9-ethylcarbazole. Finally, the disks were counterstained with hematoxylin and mounted in aqueous mounting medium. Negative disks were not incubated with anti-BrdU antibody. Cells with

red-stained nuclei by 3-amino-9-ethylcarbazole were identified as cells with BrdU incorporation. The BrdU labeling index (percentage) was determined by the number of BrdU-positive nuclei in cells and analyzed and scored by two different observers under light microscopy. The experiments were done in triplicate.

In Vitro Wound Healing Migration Assays

Wound healing migration assays were performed as previously described.²¹ In brief, keratinocytes or myofibroblasts were seeded on 24-well multiplates and grown to confluence. After 60 minutes of incubation with AngII (100 pg/ml), CV-11974 (10^{-7} mol/L), CV-11974 (10^{-7} mol/L with AngII 100 pg/ml), PD-123319 (10^{-5} mol/L), PD-123319 (10^{-5} mol/L with AngII 100 pg/ml), or vehicle in the medium, respectively, the monolayers of keratinocytes or myofibroblasts were wounded with a cell scraper and photographed at different times by a phase contrast microscope equipped with Olympus Camedia C-3030ZOOM digital camera (Olympus, Tokyo, Japan). The re-epithelization index (percentage) was determined by the percentage of new epithelium present in the initial wound using image analysis software program Analytical Imaging Station for Windows (Imaging Research Inc., Willowdale, Canada). The experiments were done in triplicate.

Statistical Analysis

Statistical significance of the quantitative measurement was assessed by Turkey's multiple range test. Each time point was analyzed separately, and two-tailed α -levels of $P \leq 0.05$ are significant.

Results

Effects of AT₁-Signaling Blockade on Re-Epithelization in Vivo

The effect of AT₁ blocker on re-epithelization *in vivo* was evaluated kinetically (Figure 1, A and B). On day 12, the control group and the group with TCV-116, 1 mg/kg/day, showed 100% re-epithelization of the wound (Figure 1A). However, the group with TCV-116, 10 mg/kg/day, showed delayed re-epithelization and delayed dermal repair. At all time points, administration of TCV-116 suppressed the keratinocyte re-epithelization dose dependently (Figure 1B).

Effects of AT₁-Signaling Blockade on Vascular Growth in Vivo

The effect of AT₁ blocker on vascular growth at the wound bed was evaluated kinetically (Figure 1, C and D). On day 9 after wounding, the control group showed many blood vessels in the dermis, however, the administration of TCV-116 suppressed angiogenesis dose dependently (Figure 1C). Administration of TCV-116, 1 mg/kg/day, slightly suppressed angiogenesis at all time points ex-

cept for the significant suppression on day 9 (Figure 1D). Administration of TCV-116, 10 mg/kg/day, significantly suppressed angiogenesis at all time points.

Immunoprecipitation and Western Blot Analysis

To analyze the effects of AngII receptor signaling on keratinocytes and myofibroblasts, we examined the expression of AngII receptors using immunoprecipitation and Western blot analysis (Figure 2). The results showed the signals of both AT₁ and AT₂ in the epidermal strips, cultured keratinocytes, and myofibroblasts. The signal of AT₁ is prominent compared to that of AT₂ in the epidermal strips, keratinocytes, and myofibroblasts.

Effects of Stimulation or Blockade of AngII Receptor Signaling on BrdU Incorporation into Cultured Keratinocytes

We investigated the level of cell proliferation by using BrdU labeling index. BrdU, a thymidine analog, can be incorporated into DNA in the S phase of cell cycle.¹⁶ Hence, the cells that traversed S phase can be detected with an anti-BrdU antibody. Based on the analysis of AngII receptor expression, keratinocytes were exposed to AngII, CV-11974, CV-11974 with AngII (selective AT₂ signaling), PD-123319, PD-123319 with AngII (selective AT₁ signaling), or vehicle, respectively, for 24 hours and offered for analyzing BrdU labeling index (percentage) (Figure 3, A and B). The presence of AngII significantly enhanced ($P < 0.05$) the BrdU incorporation into keratinocytes compared to the control. The similar enhancing effect was recognized in the group of PD-123319 with AngII. In contrast, the presence of CV-11974 with AngII significantly suppressed ($P < 0.05$) the BrdU incorporation compared to the control or the group with AngII. These results suggest that AT₁ signaling accelerates and AT₂ signaling inhibits BrdU incorporation into keratinocytes, respectively.

Wound Healing Migration Assays of Cultured Keratinocytes

The re-epithelizing movements of keratinocytes were recorded starting from wounding until one of the wounds was re-epithelized (40 hours later) (Figure 3, C and D). At all time points except for 16 hours after scraping, the presence of AngII accelerated re-epithelization compared to the control. The similar accelerating effect was recognized in the group of PD-123319 with AngII. In contrast, the presence of CV-11974 with AngII significantly suppressed re-epithelization compared to the control or the group with AngII. The results suggest that AT₁ signaling accelerates and AT₂ signaling inhibits the re-epithelization of keratinocytes, respectively.

Figure 2. Immunoprecipitation and Western blot analysis of AngII receptors. In the epidermal strips, cultured keratinocytes, and myofibroblasts, the signals of both AT₁ and AT₂ were detected. The signal of AT₁ is prominent compared to that of AT₂. SM, size marker; ES, epidermal strips; CK, cultured keratinocytes; MF, myofibroblasts.

Effects of Stimulation or Blockade of AngII Receptor Signaling on BrdU Incorporation into Cultured Myofibroblasts

Based on the analysis of AngII receptor expression, myofibroblasts were exposed to AngII, CV-11974, CV-11974 with AngII (selective AT₂ signaling), PD-123319, PD-123319 with AngII (selective AT₁ signaling), or vehicle, respectively, for 24 hours and offered for analyzing BrdU labeling index (percentage) (Figure 4, A and B). The presence of AngII significantly enhanced ($P < 0.05$) the BrdU incorporation into myofibroblasts compared to the control. The similar enhancing effect was recognized in the group of PD-123319 with AngII. In contrast, the presence of CV-11974 with AngII significantly suppressed ($P < 0.05$) the BrdU incorporation compared to the control group or the group with AngII. The results suggest that AT₁ signaling enhances and AT₂ signaling suppresses the BrdU incorporation into myofibroblasts, respectively.

Wound Healing Migration Assays of Cultured Myofibroblasts

The recovering movements of myofibroblasts were recorded starting from wounding until one of the wounds was recovered (38 hours later) (Figure 4, C and D). At all of the time points except for 12 hours after scraping, the presence of AngII significantly accelerated ($P < 0.05$) recovering compared to the control. The similar accelerating effect was recognized in the group of PD-123319 with AngII. On the contrary, the presence of CV-11974 with AngII significantly inhibited ($P < 0.05$) recovering compared to the control or the group with AngII. The results suggest that AT₁ signaling accelerates and AT₂ signaling inhibits the recovering of myofibroblasts, respectively.

Discussion

It has been proposed that exogenous administration of AngII is a potent accelerator of cutaneous wound repair.^{7,8} These reports showed that topical administration of AngII could accelerate skin wound healing by stimulating dermal repair including angiogenesis and epidermal repair *in vivo*. However, the mechanism of AngII-induced acceleration of skin wound healing is poorly understood. In the present report, we studied the effects of AngII on skin wound healing focusing on AngII receptor-mediated signaling.

AngII, the effector peptide of the renin-Ang system, is now known to have growth regulating properties in various cell types.²² AngII exerts its actions by binding the specific receptors. Two major AngII receptors with the seven transmembrane domains, namely, AT₁ and AT₂, have been identified. AT₁ is expressed in the brain, kidney, liver, heart, adrenal gland, ovary, testis, and vascular smooth muscle cells.²³ AT₂ is abundantly and widely expressed in fetal tissues. Most of the AngII-induced actions are mediated by AT₁. AT₁ activates many signaling pathways and can stimulate tyrosine phosphorylation [through c-Src, Pyk2, and transactivation of epidermal growth factor (EGF) receptor (EGFR)] and activation of phospholipases and protein kinase C.²⁴ A recent study showed that AngII-induced extracellular signal-regulated kinase (ERK) activation was mediated by EGFR transactivated via AT₁.¹⁸ In contrast, AT₂ stimulates none of these (except p38 MAPK) and appears to involve protein

Figure 1. Effects of AT₁ blocker on re-epithelization and angiogenesis during skin wound healing. **A:** Histological findings of the skin wounds on day 12 after wounding (H&E staining). The control group and the group with TCV-116, 1 mg/kg, showed 100% re-epithelization. The group with TCV-116, 10 mg/kg, showed delayed re-epithelization and delayed dermal repair. The crust still attached on the surface. **Arrows** indicate the re-epithelized edges. **B:** Time course of re-epithelization. The re-epithelization index (percentage of the wound site with new epithelization) was evaluated microscopically using tissue sections stained with H&E. Rats were orally administered with vehicle or TCV-116, 1 or 10 mg/kg/day. Data are represented as mean $\pm$ SD ($n = 5$). *, Significantly different from controls ($P < 0.05$). Administration of TCV-116 suppressed the re-epithelization dose dependently and postponed the day of 100% re-epithelization. **C:** Immunohistochemical staining for von Willebrand factor of the wound bed on day 9. The control wound showed many blood vessels. Administration of TCV-116 suppressed vascular growth dose dependently. **D:** Time course of angiogenesis. Number of blood vessels was evaluated microscopically using tissue sections immunohistochemically stained for von Willebrand factor. Each time point was analyzed separately. Data are represented as mean $\pm$ SD ($n = 5$). *, Significantly different from controls ($P \leq 0.05$). Administration of TCV-116, 1 mg/kg/day, slightly (on days 3, 6, 12, and 15) or significantly (day 9) suppressed vascular growth. At all time points, administration of TCV-116, 10 mg/kg/day, significantly inhibited vascular growth. Original magnifications: $\times 10$ [**A** (top layer)]; $\times 20$ [**A** (bottom layer), **C**].

Figure 3. BrdU incorporation and wound healing migration assays of cultured keratinocytes. **A:** Detection of BrdU incorporation by immunohistochemical staining. The BrdU-labeled nuclei stained red. CV, CV-11974; PD, PD-123319. **B:** Effects of stimulation or blockade of AngII receptor signaling on BrdU incorporation. Data are represented as mean $\pm$ SD ($n = 3$). *, Significantly different from controls ($P \leq 0.05$). ♦, Significantly different from AngII-treated group ($P \leq 0.05$). AngII or PD-123319 with AngII enhanced BrdU incorporation. CV-11974 with AngII suppressed BrdU incorporation. **C:** Wound healing migration assays. The phase contrast microscopic findings at 40 hours after scraping. **D:** Results of wound healing migration assays. Data are represented as mean $\pm$ SD ($n = 3$). *, Significantly different from controls ($P \leq 0.05$). ♦, Significantly different from AngII-treated group ($P \leq 0.05$). AngII or PD-123319 with AngII accelerated re-epithelization. CV-11974 with AngII inhibited keratinocyte re-epithelization. Original magnifications: $\times 20$ (**A**); $\times 10$ (**C**).

Figure 4. BrdU incorporation and wound healing migration assays of cultured myofibroblasts. **A:** Detection of BrdU incorporation by immunohistochemical staining. The BrdU-labeled nuclei stained red. CV, CV-11974; PD, PD-123319. **B:** Effects of stimulation or blockade of AngII receptor signaling on BrdU incorporation. Data are represented as mean $\pm$ SD ($n = 3$). *, Significantly different from controls ($P \leq 0.05$). ♦, Significantly different from AngII-treated group ($P \leq 0.05$). AngII or PD-123319 with AngII enhanced BrdU incorporation. CV-11974 with AngII suppressed BrdU incorporation. **C:** Wound healing migration assays. The phase contrast microscopic findings at 36 hours after scraping. **D:** Results of wound healing migration assays. Data are represented as mean $\pm$ SD ($n = 3$). *, Significantly different from controls ($P \leq 0.05$). ♦, Significantly different from AngII-treated group ($P \leq 0.05$). AngII or PD-123319 with AngII accelerated myofibroblast recovering. CV-11974 with AngII inhibited recovering. Original magnifications: $\times 20$ (**A**); $\times 10$ (**C**).

tyrosine phosphatases (SHP-1), MAP kinases (p38 and ERK1/2), and G α s (independent of $\beta\gamma$ G proteins).

EGFR is an 1186-amino acid glycoprotein containing a single transmembrane domain, an extracellular portion involved in ligand binding, and an intracellular portion harboring the tyrosine kinase domain.²⁵ The C-terminal sequences harbor autophosphorylation sites that are important for the initiation of downstream signaling.²⁶ Stimulation of EGFR results in the simultaneous activation of multiple signaling pathways including the RAS-RAF-MEK-MAPK, the PLC- γ /PKC, the PI-3K/AKT cascades, and the JAK and STATS pathways, and these pathways are often functionally interlinked. The known mammalian ligands that act as direct agonists for EGFR include transforming growth factor (TGF)- α , heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin, epiregulin, and epigen.²⁷ These ligands are synthesized as transmembrane precursors and must therefore be proteolytically cleaved by metalloproteases to release the mature growth factor. The agonist-occupied EGFR undergoes gene transcription and mitogenesis. In addition to its cognate ligands, EGFR is activated by stimuli that do not directly interact with EGFR ectodomain including G protein-coupled receptor (GPCR) ligands, other receptor tyrosine kinase agonists, cytokines, chemokines, and cell adhesion elements.²⁶ This process is termed EGFR transactivation and the GPCR-induced EGFR transactivation is stimulated by bradykinin, endothelin (ET)-1, lysophosphatidic acid, interleukin-8, carbachol stimulation, or AngII. Literature has shown that AT₁ stimulates processing of pro-HB-EGF by metalloproteinases including a disintegrin and metalloprotease 12 (ADAM 12), and the released HB-EGF transactivates EGFR.²⁸ AngII-induced transactivation of EGFR is essential for the activation of downstream Sr/Thr kinases such as ERK and subsequent protein synthesis.²⁹

Our *in vivo* study showed that administration of AT₁ blocker suppressed rat skin wound healing by inhibiting re-epithelization, dermal repair, and angiogenesis. The process of re-epithelization consists of keratinocyte migration and proliferation. Keratinocytes undergo profound changes in morphology as they migrate.³⁰ The cytoskeleton is critical to these changes and cell migration. EGF-induced activation of PLC- γ stimulates cell motility by releasing PIP₂-bound gelsolin from the membrane, thereby restoring its ability to bind, sever, and cap polymerized actin filaments, a process required for filopodia/lamellipodia extension and retraction in motile cells.^{31,32} Thus, EGFR-mediated activation of PLC- γ is believed to be critical for the reorganization of the actin cytoskeleton and contribute to the initiation of the asymmetric motile phenotype.³³

For keratinocytes to migrate, they need to detach themselves from the basal lamina to which they are attached through the hemidesmosomes, anchoring contacts linking laminin via integrin $\alpha 6\beta 4$ to the keratinocyte's keratin filament network.³⁴ A fraction of EGFR combines with the hemidesmosomal integrin $\alpha 6\beta 4$ in normal keratinocytes.³⁵ Activation of EGFR causes tyrosine phosphorylation of the $\beta 4$ cytoplasmic domain by activating

Fyn and disruption of hemidesmosomes and releasing keratinocytes for migration.

The process of re-epithelization is also impaired without the appropriate expression and action of enzymes needed to dissolve substrates and matrix materials for keratinocytes to migrate. Collagenase-1 (matrix metalloproteinase-1) is needed for keratinocyte migration on type I collagen and is up-regulated in keratinocytes at the very edge of the wound.³⁰ Keratinocyte contact with type I collagen is sufficient to induce collagenase-1 expression, whereas sustained enzyme production requires EGFR activation by HB-EGF as an obligatory intermediate step, thereby maintaining collagenase-1-dependent migration during the re-epithelization.³⁶

Autocrine keratinocyte-derived EGFR ligands including TGF- α , amphiregulin, and HB-EGF contribute to keratinocyte proliferation.³⁷ Literature demonstrated that EGFR activation is indispensable for DNA synthesis³⁸ and cell cycle progression from the G₁ to S phase³⁹ in cultured keratinocytes and suggests an essential role of EGFR activation in keratinocyte proliferation.

These data suggest that EGFR activation is important for keratinocyte re-epithelization including migration and proliferation. Accelerated epidermal repair by topical administration of AngII *in vivo*^{7,8} may be because of induction of AT₁ signaling and at least in part because of transactivation of EGFR. Consistent with this notion, our experiments *in vivo* showed that administration of AT₁ blocker suppressed keratinocyte re-epithelization during skin wound healing. *In vitro* experiments showed that induction of AT₁ signaling enhanced keratinocyte BrdU incorporation and keratinocyte re-epithelization in wound healing migration assays. In contrast, induction of AT₂ signaling acted vice versa. Therefore, we conclude that AT₁ signaling accelerates keratinocyte proliferation and migration and AT₂ signaling suppresses keratinocyte proliferation and migration.

The process by which fibroblasts leave their collagen-rich environment to enter the wound site requires them to modify their behavior and characteristics.³⁰ Early during injury, contraction of dermal myofibroblasts rapidly reduces dermal defects and decreases the area requiring re-epithelization. Later in the injury response, dermal myofibroblasts are stimulated to migrate into the wound and proliferate. AngII increases the TGF- $\beta 1$ gene and protein output in cardiac fibroblasts and, especially, in myofibroblasts.⁴⁰ In cardiac fibroblasts, AngII via AT₁ and TGF- $\beta 1$ increased fibronectin secretion, smooth muscle α -actin synthesis, and formation of actin stress fibers and enhanced attachment of fibroblasts to a fibronectin matrix and thereby stimulated and modified fibroblasts into a myofibroblast-like phenotype.⁴¹ The blockade of AT₁ signaling impaired fibroblast proliferation, consequent differentiation into myofibroblasts, and the synthesis of TGF- $\beta 1$.⁴² These data suggest AngII via AT₁ stimulates TGF- $\beta 1$ expression, thereby resulting in modification of fibroblast phenotype into myofibroblasts and contributing tissue re-modeling in wound healing. A recent study showed that the endothelial cell-derived vasoconstrictor ET-1 stimulates colonic subepithelial myofibroblast contraction and migration via ET receptor

mediated myosin phosphorylation that may be regulated by both calcium and rhoA signaling pathways.⁴³ AngII via AT₁ stimulates ET-1 gene expression⁴⁴ and RAS/RAF/ERK pathways are at least in part involved in AngII-induced proliferation and ET-1 gene expression in rat cardiac fibroblasts⁴⁵ and aortic smooth muscle cells.⁴⁶ These data suggest that induction of AT₁ signaling is important for dermal repair including transformation of fibroblasts into myofibroblasts. Supporting the notion, our experiments showed that induction of AT₁ signaling enhanced myofibroblast BrdU incorporation and myofibroblast recovering in wound healing migration assays. In contrast, induction of AT₂ signaling acted vice versa. Therefore, we conclude that AT₁ signaling accelerates myofibroblast proliferation and migration and AT₂ signaling suppresses myofibroblast proliferation and migration. Recent studies have shown that AngII via AT₁ contributes fibrotic response after tissue injury.⁴⁷ Inhibiting AT₁ signaling through the use of AT₁ blocker or AngII-converting enzyme inhibitor has proven very useful in the treatment of cardiac hypertrophy⁴⁷ or renal failure⁴⁸ because they prevent pathological fibrosis. In the skin, overinduction of AT₁ signaling may contribute to cause the pathological fibrotic response such as scarring.

In the experiments using NIH3T3 fibroblasts that were transiently co-transfected with rat AT₁ or AT₂ expression vectors, the presence of AngII enhanced or reduced the EGFR-induced mitogen activated proliferation kinase (MAPK) activation in the cells expressing AT₁ or AT₂, respectively.⁴⁹ In the cells expressing both AngII subtype receptors, AngII stimulated an enhancement of EGFR-induced MAPK activation. Consistent with the results, our experiments indicated that the presence of AngII resulted in induction of AT₁ signaling in keratinocytes and myofibroblasts expressing both AngII receptors.

Another aspect for wound healing is neovascularization. Endothelial cell migration and tube formation in response to vascular endothelial growth factor (VEGF) play an important role in the process of angiogenesis. AngII via AT₁ stimulates processing of pro-HB-EGF by metalloproteinases, and the released HB-EGF transactivates EGFR to induce angiogenesis via the combined effect of angiopoietin-2 and VEGF, whereas AT₂ attenuates them by blocking EGFR phosphorylation in cardiac microvascular endothelial cells expressing both AT₁ and AT₂.² Stimulation of AT₂ signaling suppresses the phosphorylation of Akt and its downstream effector endothelial NO synthase that is pivotal to VEGF-induced angiogenesis and inhibits VEGF-induced endothelial cell migration and tube formation of endothelial cells.⁵⁰ Recent studies demonstrated a powerful capacity of VEGF to increase AngII-converting enzyme in human umbilical vein endothelial cells, suggesting a synergistic relationship between renin Ang system and VEGF.⁵¹ ET-1 is secreted not only from endothelial cells but also from all rat vascular smooth muscle cells⁵² and induces angiogenic responses through ET(B) receptor and that stimulates neovascularization in concert with VEGF in cultured endothelial cells.⁵³ TGF- β 1 stimulates ET-1 secretion from vascular smooth muscle cells and endothelial cells.⁵² These data suggest that AngII stimulates vascu-

lar growth via AT₁ in skin wound healing. Consistent with this notion, the results of our study showed that blockade of AT₁ signaling suppressed angiogenesis during skin wound healing *in vivo*. Taken together, AT₁ signaling accelerates proliferation and migration of both keratinocytes and myofibroblasts and AT₂ signaling suppresses these effects. In other words, skin wound healing is regulated by the balance of opposing signals between AT₁ and AT₂.

References

1. Gyurko R, Kimura B, Kurian P, Crews FT, Phillips MI: Angiotensin II receptor subtypes play opposite roles in regulating phosphatidylinositol hydrolysis in rat skin slices. *Biochem Biophys Res Commun* 1992, 186:285–292
2. Fujiyama S, Matsubara H, Nozawa Y, Maruyama K, Mori Y, Tsutsumi Y, Masaki H, Uchiyama Y, Koyama Y, Nose A, Iba O, Tateishi E, Ogata N, Jyo N, Higashiyama S, Iwasaka T: Angiotensin AT₁ and AT₂ receptors differentially regulate angiopoietin-2 and vascular endothelial growth factor expression and angiogenesis by modulating heparin binding-epidermal growth factor (EGF)-mediated EGF receptor transactivation. *Circ Res* 2001, 88:22–29
3. Shibasaki Y, Matsubara H, Nozawa Y, Mori Y, Masaki H, Kosaki A, Tsutsumi Y, Uchiyama Y, Fujiyama S, Nose A, Iba O, Tateishi E, Hasegawa T, Horiuchi M, Nahmias C, Iwasaka T: Angiotensin II type 2 receptor inhibits epidermal growth factor receptor transactivation by increasing association of SHP-1 tyrosine phosphatase. *Hypertension* 2001, 38:367–372
4. Murasawa S, Mori Y, Nozawa Y, Gotoh N, Shibuya M, Masaki H, Maruyama K, Tsutsumi Y, Moriguchi Y, Shibasaki Y, Tanaka Y, Iwasaka T, Inada M, Matsubara H: Angiotensin II type 1 receptor-induced extracellular signal-related protein kinase activation is mediated by Ca²⁺/calmodulin-dependent transactivation of epidermal growth factor receptor. *Circ Res* 1998, 82:1338–1348
5. Eguchi S, Numaguchi K, Iwasaki H, Matsumoto T, Yamakawa T, Utsumono H, Motley ED, Kawakatsu H, Owada KM, Hirata Y, Marumo F, Inagami T: Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *J Biol Chem* 1998, 273:8890–8896
6. Rodgers KE, Abiko M, Giegis W, St. Amand K, Campeau J, diZerega G: Acceleration of dermal tissue repair by angiotensin II. *Wound Repair Regen* 1997, 5:175–183
7. Rodgers KE, Xiong S, Felix JC, Roda R, Espinosa T, Maldonado S, diZerega G: Development of angiotensin (1-7) as an agent to accelerate dermal regeneration. *Wound Repair Regen* 2001, 9:241–250
8. Rodgers KE, Roda N, Felix JC, Espinosa T, Maldonado S, diZerega G: Histological evaluation of the effects of angiotensin peptides on wound repair in diabetic mice. *Exp Dermatol* 1997, 5:175–183
9. Sun Y, Weber KT: Angiotensin-converting enzyme and wound healing in diverse tissue of the rat. *J Lab Clin Med* 1996, 127:94–101
10. Abiko M, Rodgers KE, Campeau JD, Nakamura R, Dizerega GS: Alterations of angiotensin II receptor levels in sutured wounds in rat skin. *J Invest Surg* 1996, 9:447–453
11. Kimura B, Sumners C, Phillips MI: Changes in skin angiotensin II receptors in rats during wound healing. *Biochem Biophys Res Commun* 1992, 187:1087–1090
12. Viswanathan M, Saavedra JM: Expression of angiotensin II AT₂ receptors in the rat skin during experimental wound healing. *Peptides* 1992, 13:783–786
13. Gyurko R, Kimura B, Kurian P, Crews FT, Phillips MI: Angiotensin II receptor subtypes play opposite roles in regulating phosphatidylinositol hydrolysis in rat skin slices. *Biochem Biophys Res Commun* 1992, 186:285–292
14. Yoshimura M, Yasue H: Hemodynamic and humoral effects of angiotensin II antagonist, candesartan cilexetil, in patients with congestive heart failure. *Cardiology* 2000, 93:175–182
15. Giorno R: A comparison of two immunoperoxidase staining methods

- based on the avidin-biotin interaction. *Diagn Immunol* 1984, 2:161–166
16. Kondo S, Hozumi Y, Mitsuhashi Y: Comparative inhibitory effects of vitamin D3 and an analogue on normal psoriatic epidermis in organ culture. *Arch Dermatol Res* 2000, 292:550–555
17. Oda D, Gown AM, Vande Berg JS, Stern R: Instability of the myofibroblast phenotype in culture. *Exp Mol Pathol* 1990, 52:221–234
18. Murasawa S, Mori Y, Nozawa Y, Gotoh N, Shibuya M, Masaki H, Maruyama K, Tsutsumi Y, Moriguchi Y, Shibasaki Y, Tanaka Y, Iwasaka T, Inada M, Matsubara H: Angiotensin II type 1 receptor-induced extracellular signal-related protein kinase activation is mediated by Ca²⁺/calmodulin-dependent transactivation of epidermal growth factor receptor. *Circ Res* 1998, 82:1338–1348
19. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, 227:680–685
20. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1973, 76:4350–4354
21. Penas PF, Gomez M, Buezo GF, Rios L, Yanez-Mo M, Cabanas C, Sanchez-Madrid F, Garcia-Diez A: Differential expression of activation epitopes of beta1 integrins in psoriasis and normal skin. *J Invest Dermatol* 1998, 111:19–24
22. Timmermans PB, Wong PC, Chiu AT, Herblin WF, Benfield P, Carini DJ, Lee RJ, Wexler RR, Saye JA, Smith RD: Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol Rev* 1993, 45:205–251
23. Burnier M, Brunner HR: Angiotensin II receptor antagonists. *Lancet* 2000, 355:637–645
24. Berk BC: Angiotensin type 2 receptor (AT2R): a challenging twin. *Sci STKE* 2003, 181:16
25. Wells A: EGF receptor. *Int J Biochem* 1999, 31:637–643
26. Gschwind A, Zwick E, Prenzel N, Lessner M, Ullrich A: Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. *Oncogene* 2001, 20:1594–1600
27. Harris RC, Chung E, Coffey RJ: EGF receptor ligands. *Exp Cell Res* 2003, 284:2–13
28. Asakura M, Kitakaze M, Takashima S, Liao Y, Ishikura F, Yoshinaka T, Ohmoto H, Node K, Yoshino K, Ishiguro H, Asanuma H, Sanada S, Matsumura Y, Takeda H, Beppu S, Tada M, Hori M, Higashiyama S: Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. *Nat Med* 2002, 8:35–40
29. Eguchi S, Inagami T: Signal transduction of angiotensin II type1 receptor through receptor tyrosine kinase. *Regul Peptides* 2000, 91:13–20
30. Falanga V: Mechanisms of cutaneous wound repair. *Fitzpatrick's Dermatology in General Medicine*. Edited by IM Freedberg, AZ Eizen, K Wolff, KF Austen, LA Goldsmith, SI Katz. New York, McGraw-Hill, 2003, pp 236–246
31. Chen P, Murphy-Ullrich JE, Wells A: A role for gelsolin in actuating epidermal growth factor-mediated cell motility. *J Cell Biol* 1996, 134:689–698
32. van Leeuwen FN, van Delft S, Kain HE, van der Kammen RA, Collard JG: Rac regulates phosphorylation of the myosin-II heavy chain, actinomyosin disassembly and cell spreading. *Nat Cell Biol* 1999, 1:242–248
33. Wells A, Gupta K, Chang P, Swindle S, Glading A, Shiraha H: Epidermal growth factor receptor-mediated motility in fibroblasts. *Microsc Res Tech* 1998, 43:395–411
34. Krawczyk WS, Wilgram GF: Hemidesmosome and desmosome morphogenesis during epidermal wound healing. *J Ultrastruct Res* 1973, 45:93–101
35. Mariotti A, Kedeshian PA, Dans M, Curatola AM, Gagnoux-Palacios L, Giancotti FG: EGF-R signaling through Fyn kinase disrupts the function of integrin alpha6beta4 at hemidesmosomes: role in epithelial cell migration and carcinoma invasion. *J Cell Biol* 2001, 155:447–458
36. Pilcher BK, Dumin J, Schwartz MJ, Mast BA, Schultz GS, Parks WC, Welgus HG: Keratinocyte collagenase-1 expression requires an epidermal growth factor receptor autocrine mechanism. *J Biol Chem* 1999, 274:10372–10381
37. Dlugosz AA, Cheng C, Williams EK, Darwiche N, Dempsey PJ, Mann B, Dunn AR, Coffey Jr R, Yuspa SH: Autocrine transforming growth factor alpha is dispensable for v-rasHa-induced epidermal neoplasia: potential involvement of alternate epidermal growth factor receptor ligands. *Cancer Res* 1995, 163:257–265
38. Coffey Jr RJ, Sipes NJ, Bascom CC, Graves-Deal R, Pennington CY, Weissman BE, Moses HL: Growth modulation of mouse keratinocytes by transforming growth factors. *Cancer Res* 1998, 48:1596–1602
39. Kobayashi T, Hashimoto K, Okumura H, Asada H, Yoshikawa K: Endogenous EGF-family growth factors are necessary for the progression from the G1 to S phase in human keratinocytes. *J Invest Dermatol* 1998, 111:616–620
40. Campbell SE, Katwa LC: Angiotensin II stimulated expression of transforming growth factor-beta 1 in cardiac fibroblasts and myofibroblasts. *J Mol Cell Cardiol* 1997, 29:1947–1958
41. Thibault G, Lacombe MJ, Schnapp LM, Lacasse A, Bouzeghrane F, Lapalme G: Upregulation of alpha(8)beta(1)-integrin in cardiac fibroblast by angiotensin II and transforming growth factor-beta1. *Am J Physiol* 2001, 281:C1457–C1467
42. Klahr S, Morrissey JJ: Comparative study of ACE inhibitors and angiotensin II receptor antagonists in interstitial scarring. *Kidney Int Suppl* 1977, 63:S111–S114
43. Kernochan LE, Tran BN, Tangkijvanich P, Melton AC, Tam SP, Yee Jr HF: Endothelin-1 stimulates human colonic myofibroblast contraction and migration. *Gut* 2002, 50:65–70
44. Fujisaki H, Ito H, Hirata Y, Tanaka M, Hata M, Liu M, Adachi S, Akimoto H, Marumo F, Hiroe M: Natriuretic peptides inhibit angiotensin II-induced proliferation of rat cardiac fibroblasts by blocking endothelin-1 gene expression. *J Clin Invest* 1995, 96:1059–1065
45. Cheng TH, Cheng PY, Shih NL, Chen IB, Wang DL, Chen JJ: Involvement of reactive oxygen species in angiotensin II-induced endothelin-1 gene expression in rat cardiac fibroblasts. *J Am Coll Cardiol* 2003, 42:1485–1494
46. Hong HJ, Chan P, Liu JC, Juan SH, Huang MT, Lin JG, Cheng TH: Angiotensin II induces endothelin-1 gene expression via extracellular signal-regulated kinase pathway in rat aortic smooth muscle cells. *Cardiovasc Res* 2004, 61:159–168
47. Miura S, Saku K, Karnik SS: Molecular analysis of the structure and function of the angiotensin II type 1 receptor. *Hypertens Res* 2003, 26:937–943
48. Shusterman N: Risk-benefit assessment of angiotensin II receptor antagonists. *Expert Opin Drug Safety* 2002, 1:137–152
49. De Paolis P, Porcellini A, Savoia C, Lombardi A, Gigante B, Frati G, Rubattu S, Musumeci B, Volpe M: Functional cross-talk between angiotensin II and epidermal growth factor receptors in NIH3T3 fibroblasts. *J Hypertens* 2002, 20:693–699
50. Benndorf R, Boger RH, Ergun S, Steenpass A, Wieland T: Angiotensin II type 2 receptor inhibits vascular endothelial growth factor-induced migration and in vitro tube formation of human endothelial cells. *Circ Res* 2003, 93:438–447
51. Saijonmaa O, Nyman T, Kosonen R, Fyhrquist F: Upregulation of angiotensin-converting enzyme by vascular endothelial growth factor. *Am J Physiol* 2001, 280:H885–H891
52. Sugo S, Minamino N, Shoji H, Isumi Y, Nakao K, Kangawa K, Matsuo H: Regulation of endothelin-1 production in cultured rat vascular smooth muscle cells. *J Cardiovasc Pharmacol* 2001, 37:25–40
53. Salani D, Taraboletti FG, Rosano L, Di Castro V, Borsotti P, Giavazzi R, Bagnato A: Endothelin-1 induces an angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. *Am J Pathol* 2000, 157:1703–1711