A Novel Function of Angiotensin II in Skin Wound Healing

**INDUCTION OF FIBROBLAST AND KERATINOCYTE MIGRATION BY ANGIOTENSIN II VIA HEPARIN-BINDING EPIDERMAL GROWTH FACTOR (EGF)-LIKE GROWTH FACTOR-MEDIATED EGF RECEPTOR TRANSACTIVATION**

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Yoshihiro Yahata, Yuji Shirakata, Sho Tokumaru, Lujun Yang, Xiuju Dai, Mikiko Tohyama, Teruko Tsuda, Koji Sayama, Masaru Iwai, Masatsugu Horiuchi, and Koji Hashimoto

From the Department of Dermatology and the Division of Medical Biochemistry and Cardiovascular Biology, Ehime University School of Medicine, Shitsukawa, Toon, Ehime 791-0295, Japan

The role of angiotensin II (Ang II) in the control of systemic blood pressure and volume homeostasis is well known and has been extensively studied. Recently, Ang II was suggested to also have a function in skin wound healing. In the present study, the in vivo function of Ang II in skin wound healing was investigated using Ang II type 1 receptor (AT1R) knock-out mice. Wound healing in these mice was found to be markedly delayed. Keratinocytes and fibroblasts play important roles in wound healing, and thus the effect of Ang II on the migration of these cells was examined. Ang II stimulated keratinocyte and fibroblast migration in a dose-dependent manner. It has been reported that G protein-coupled receptor (GPCR) activation induces epidermal growth factor (EGF) receptor (EGFR) transactivation through the shedding of heparin-binding EGF-like growth factor (HB-EGF). As AT1R is a GPCR, it was hypothesized that Ang II-induced keratinocyte and fibroblast migration is mediated by EGFR transactivation. Ang II induced EGFR phosphorylation, which was inhibited by an AT1R antagonist, HB-EGF neutralizing antibody, and an HB-EGF antagonist in both keratinocytes and in fibroblasts. Moreover, Ang II-induced migration of keratinocytes and fibroblasts was also prevented by these inhibitors. Taken together, these findings clearly demonstrate, for the first time, that Ang II plays an important role in skin wound healing and that it functions by accelerating keratinocyte and fibroblast migration in a process mediated by HB-EGF shedding.

Cutaneous wound healing requires precise coordination of epithelialization, dermal repair, and angiogenesis (1). In turn, epithelialization is ultimately dependent on the migratory, proliferative, and differentiation abilities of keratinocytes, while dermal repair requires the production of extracellular matrix by fibroblasts. The growth and differentiation of these two cell types are regulated by several different growth factors (2).

The vasoactive octapeptide angiotensin II (Ang II) has a well-described role in the control of systemic blood pressure and volume homeostasis. In the rat, both cardiac and skin fibroblasts express Ang II receptors, which have been shown to be involved in cell growth and the activation of second messenger pathways, such as the mobilization of intracellular calcium (3, 4). In addition, Ang II was shown to act as a mitogen for smooth muscle cells, fibroblasts, and endothelial cells (5–10). Mammalian cells express two types of Ang II receptors, Ang II type 1 receptor (AT1R) and Ang II type 2 receptor (AT2R) (11). Most of the known biological effects of Ang II are mediated through AT1R, a G protein-coupled receptor (GPCR) that is expressed in a wide variety of cells and tissues. In mouse, there are two isoforms of AT1R, AT1aR and AT1bR. AT1R has been shown to mediate mitogenesis in cardiac fibroblasts and vascular smooth muscle cells (12, 13), whereas in fibroblasts, the activation of AT2R has two opposing effects, inhibition of cell growth (14, 15) and promotion of apoptosis (16). Thus, the balance between the expressions of these two receptor types may be crucial in determining the response to Ang II. The integral role of Ang II in regulating systemic blood pressure and volume homeostasis is well known and has been extensively studied. In the present study, we show that Ang II is also involved in wound repair.

Recent investigations demonstrated that the stimulation of GPCR induces shedding of epidermal growth factors (EGF) via the activation of a disintegrin and metalloprotease (ADAM), followed by transactivation of the EGF receptor (EGFR). HB-EGF, an EGF family member, is thought to play a major role in this process. HB-EGF is a single transmembrane-spanning protein that is proteolytically cleaved at a juxtamembrane site, leading to the shedding of soluble EGFR ligand, which in turn activates the EGFR in an autocrine/paracrine manner (17).

The physiological functions as well as the underlying cellular and molecular mechanisms of AT1R in the cardiovascular system have been the focus of many studies, whereas the role of Ang II receptors in skin is not well established. It has been reported that adult rat skin contains predominantly AT1R (18) and that Ang II accelerates the closure of thermal injuries and full-thickness dermal lesions. Both of these responses are associated with an enhancement of several physiological processes necessary for skin wound repair, such as the proliferation of keratinocytes and the production of extracellular matrix (19–21).

As AT1R is a GPCR and as HB-EGF plays an important role in skin wound healing, we investigated the involvement of Ang II AT1R in skin wound healing. Our results clearly demonstrated that Ang II participates in skin wound healing by accelerating both keratinocyte and fibroblast migration in a process mediated by HB-EGF shedding.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Reagents—Normal human epidermal keratinocytes were cultured in MCDB 153 type II medium as described previously (22). Keratinocytes in their fourth passage were used in this study. Nor-
normal human dermal fibroblasts were isolated from normal human skin and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (FCS). Fourth- or fifth-passage cells were used. Mouse dermal fibroblasts were isolated from neonatal wild-type and AT1aR−/− mice and cultured in DMEM/10% FCS. Fourth-passage cells were used. Recombinant Ang II, PD123319, and CRM197 were purchased from Sigma. Valsartan, an AT1 receptor-selective blocker, was provided by Novartis Pharma AG (Basel, Switzerland). Anti-HB-EGF neutralizing antibody was purchased from R&D Systems (Abingdon, England). Anti-phospho-EGFR; Transduction Laboratories (Lexington, KY) were incubated with the membranes at 4 °C overnight. Fluorescein-labeled goat anti-mouse or anti-rabbit IgG (GE Healthcare Biosciences Corp.) was used as the secondary antibody. The signal was amplified with an anti-fluorescein antibody followed by a fluorescent substrate, AttoPhos (GE Healthcare Biosciences Corp.). The membrane was scanned using FluoroImager (GE Healthcare Biosciences Corp.), and band intensity was quantified with ImageQuant™ (GE Healthcare Biosciences Corp.). The control signal was defined as one unit.

**RT-PCR**—Total RNA was extracted from cultured keratinocytes and fibroblasts using ISOGEN (Nippon Gene, Tokyo, Japan). Human AT1R and AT2R mRNAs and mouse AT1a, AT1b, and AT2R mRNAs were analyzed by RT-PCR using the primers listed in Table 1. RT-PCR was performed using RT-PCR High Plus (Toyobo Co., Ltd, Osaka, Japan) according to the manufacturer’s instructions. The cDNA was reverse-transcribed from total RNA for 30 min at 60 °C and was heated to 94 °C for 2 min. The amplification was performed using a DNA thermal cycler (Astec, Fukuoka, Japan) for 25 cycles of denaturation for 1 min at 94 °C and annealing and primer extension for 1.5 min at 60 °C.

**Migration Assay**—Migration was evaluated using a modified Boyden chamber assay, as described previously (23). Briefly, a Nucleopore polycarbonate filter (8-μm; Neuro Probe, Inc., Gaithersburg, MD) was coated with type I collagen (Nitta Gelatin, Tokyo, Japan) for 30 min at room temperature and allowed to air dry. The filter was placed on a 48-well chamber containing various concentrations of Ang II in MCDB 153 without bovine pituitary extract for keratinocytes and in DMEM/2% FCS for fibroblasts. After trypsinization, 1 × 10^6 cells in 50 μl of serum-free MCDB 153 or DMEM were added to the wells in the upper chamber. The chamber was then placed in a humidified incubator at 37 °C for 7 h, after which the upper surface of the filter was scraped to remove non-migratory cells. The filter was subsequently fixed in buffered formalin for 30 min, washed with phosphate-buffered saline, and stained with hematoxylin and eosin. The total number of cells per well was counted by microscopy.

**Proliferation Assay**—Keratinocytes or fibroblasts were seeded on 6-well plates (5 × 10^4 cells/well). Next day, medium was replaced to basal medium (MCDB 153 for keratinocytes, DMEM/2% FCS for fibroblasts), and various concentrations of Ang II were added to the medium. After 4 days cell number was counted using Coulter Counter (Z1, Coulter).

**Western Blot Analysis**—Cells were harvested by scraping with extraction buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.4), and protease inhibitors. Equal amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Primary antibodies (anti-EGFR and anti-phospho-EGFR; Transduction Laboratories, Lexington,
AT1aR−/− mice (Fig. 1A). On day 10, the wounds of wild-type mice were almost healed, whereas the wounds of AT1aR−/− mice were still about 50% their initial size. The differences in wound healing between wild-type and AT1aR−/− mice from day 2 to day 10 were significant (Fig. 1B). These results indicate that Ang II plays an important role in cutaneous wound healing.

Ang II Receptor Expression in Cultured Skin Keratinocytes and Fibroblasts—Keratinocytes and fibroblasts are the major cells responsible for cutaneous wound healing. We therefore examined which type of Ang II receptors are expressed in cultured human keratinocytes and fibroblasts. RT-PCR demonstrated that keratinocytes expressed AT1R but not AT2R mRNA, whereas fibroblasts expressed mRNA of both AT1R and AT2R (Fig. 2A). In cultured mouse keratinocytes, AT1R, but not AT2R, was expressed, while in mouse fibroblasts AT1aR, AT1bR, and AT2R were detected (Fig. 2B). These results demonstrate that Ang II receptors are expressed differentially depending on the cell type.

Angiotensin II-induced Keratinocyte and Fibroblast Proliferation and Migration—As cell migration and cell proliferation play central roles in cutaneous wound healing, we examined the effect of Ang II on cell

![FIGURE 2. Angiotensin II receptor expression in keratinocytes and fibroblasts. Angiotensin II receptor expression was analyzed by RT-PCR. A, human; B, mouse. Kc, keratinocyte; Fb, fibroblast.](image)

![FIGURE 3. Proliferation and migration of keratinocytes and fibroblasts induced by angiotensin II. Proliferation was examined by growth assay. Keratinocytes or fibroblasts were seeded on 6-well plates (5 × 10^4 cells/well), and various concentrations of Ang II were added to the medium. After 4 days cell number was counted using Coulter Counter (Z1, Coulter). A, keratinocytes; B, fibroblasts. Migration was assessed by Boyden chamber assay. C, Ang II induced keratinocyte migration by a maximum of 1.7-fold at 10^{-7} M. EGF was used as a positive control. D, Ang II induced fibroblast migration by a maximum of 2.0-fold at 10^{-7} M. PDGF was used as a positive control. *, p < 0.05.](image)
proliferation. Keratinocytes and fibroblasts were seeded on 6-well plates (5 x 10^4 cells/well), and various concentrations of Ang II (1 x 10^{-8} to 10^{-5} M) were added to the medium. After 4 days incubation, cell number was counted. Ang II did not promote cell proliferation of both type of cells (Fig. 3, A and B). Next the effect of Ang II on the migration of keratinocytes and fibroblasts was examined. Human keratinocytes and fibroblasts were seeded on type I collagen-coated filters, and various concentrations of Ang II were added to the medium. After 7 h, Ang II enhanced keratinocyte migration in a dose-dependent manner, by a maximum of 1.7-fold at 10^{-7} M compared with control (Fig. 3 C). Ang II also enhanced fibroblast migration by a maximum of 2.0-fold at 10^{-7} M compared with control (Fig. 3 D). Therefore, Ang II at a concentration of 10^{-7} M was used to stimulate keratinocytes and fibroblasts in the following experiments.

The effect of AT1R and AT2R inhibitors on Ang II-induced keratinocyte and fibroblast migration was investigated. Valsartan, a specific inhibitor for AT1R, inhibited Ang II-induced keratinocyte migration by 80%. As expected, PD123319, a specific inhibitor for AT2R, did not inhibit Ang II-induced keratinocyte migration (Fig. 4A) because these cells lack this receptor. Similar to the results in keratinocytes, valsartan inhibited Ang II-induced fibroblast migration by 76%, whereas PD123319 did not prevent Ang II-induced fibroblast migration (Fig. 4B). We further confirmed that Ang II-induced migration was mediated via AT1R signaling using fibroblast prepared from wild-type (C) and AT1aR-/— (D) mice, and Ang II-induced migration was examined. PDGF was used as a positive control. *, p < 0.05.

A Role for HB-EGF-mediated EGFR Transactivation in Ang II-induced Cell Migration—Recent studies demonstrated that the stimulation of GPCR induces the shedding of EGFs via ADAM, with subsequent transactivation of EGFR. It is well known that EGFR activation triggers cell migration (25). As AT1R is a GPCR, the phosphorylation of EGFR in response to stimulation with Ang II was investigated in both cell types. EGFR was phosphorylated 5–15 min after the addition of Ang II in keratinocytes, and Ang II-induced EGFR phosphorylation was completely blocked by pre-treatment with valsartan (Fig. 5A). In fibroblasts, the amount of phosphorylated EGFR was increased remarkably 30 min after the addition of Ang II, and phosphorylation was completely suppressed by valsartan (Fig. 5B). The AT2R blocker PD123319 did not affect Ang II-induced EGFR phosphorylation (data not shown).
The role of HB-EGF in Ang II-induced cell migration was determined by assaying keratinocyte and fibroblast migration using an anti-HB-EGF antibody. Pretreatment with the anti-HB-EGF antibody inhibited Ang II-induced keratinocyte and fibroblast migration by 100 and 48%, respectively. (Fig. 6, A and B). The effect of HB-EGF on Ang II-induced migration was further confirmed using CRM197, a non-toxic mutant of diphtheria toxin that selectively binds and inactivates HB-EGF. The addition of CRM197 into the medium inhibited Ang II-induced keratinocyte migration by 45% (Fig. 6C) and Ang II-induced fibroblast migration by 90% compared with controls (Fig. 6D).

Finally we investigated the effects of MMP inhibitor and ErbB inhibitor on Ang II-induced migration. Treatment with ErbB kinase inhibitor (AG1478; 30 nM) inhibited Ang II-induced keratinocyte and fibroblast migration completely. (Fig. 7A, B). The addition of GM6001 (MMP inhibitor) into the medium inhibited Ang II-induced keratinocyte migration by 65% (Fig. 7C) and Ang II-induced fibroblast migration by 90% compared with controls (Fig. 7D). These results indicated that Ang II-induced keratinocyte and fibroblast migration is mediated through EGFR transactivation, mainly by HB-EGF shedding.

**DISCUSSION**

The results of this study demonstrate that Ang II promotes cutaneous wound healing. While it has long been recognized that growth factors participate in cutaneous wound healing, it is remarkable that Ang II, which plays a role in the control of systemic blood pressure and volume homeostasis, is also involved in wound healing. Previous studies have shown that Ang II affects many aspects of wound repair, including cellular proliferation, chemotaxis, extracellular matrix production, and angiogenesis (20).

It has been reported that adult rat skin contains predominantly AT1R (18) and that Ang II accelerates the closure of thermal injuries and full-thickness dermal lesions in these animals. In addition to the contribution of exogenous Ang II to wound repair, studies have shown that increased levels of Ang II and ATRs are produced at the site of wound
repair (18–21). Takeda et al. (26) studied the effect of Ang II on skin wound healing and found that it is regulated by the balance of AT1R and AT2R and that the induction of AT1R signaling accelerated keratinocyte re-epithelialization and myofibroblast recovery. These reports led us to investigate the role of Ang II in skin wound healing and its mechanisms. Using AT1aR knock-out mice, we were able to show, for the first time, that Ang II is involved in cutaneous wound healing.

Skin wound healing is a complex process requiring several coordinated events, including inflammation, cell migration, cell proliferation, matrix production, and angiogenesis (1), and it involves a complex array of cells, growth factors, cytokines, and matrix components. Members of the EGF family are the most important growth factors related to epithelialization during cutaneous wound healing. Among the EGF family members, TGF-α, HB-EGF, amphiregulin, and epiregulin are autocrine growth factors produced by epidermal keratinocytes (27–30). No differences were found in the healing of either excisional dorsal wounds or ear-punch wounds between TGF-α knock-out mice and wild-type mice (31, 32). Epiregulin knock-out mice also did not show any delay in wound healing (33). The absence of an effect on wound healing in EGFR-ligand knock-out mice is probably attributable to the known functional redundancy among EGF family members. Recently, we used keratinocyte-specific-HB-EGF knock-out mice to demonstrate that HB-EGF enhances keratinocyte migration and is thus an important growth factor in skin wound healing (34). HB-EGF binds to ErbB1 and ErbB4 (35). ErbB1 knock-out mice do not exhibit impaired wound healing (36). Together, ErbB4 signaling from HB-EGF in skin wound healing should be clarified in future.

As not only proliferation but also cellular migration are essential to wound healing, the effect of Ang II on cell migration was examined. We demonstrated that the major determinant of accelerated cutaneous wound repair by Ang II is increased keratinocyte and fibroblast migration. In further experiments, cross-talk between Ang II and EGF family members, especially HB-EGF, was studied. Ang II is known to induce EGFR phosphorylation, a process referred to as EGFR transactivation. EGFR transactivation by GPCR agonists was initially thought to be independent of EGFR ligands and achieved solely by intracellular events. This interpretation was based on the rapid onset of EGFR phosphorylation as well as an apparent lack of EGFR ligands in the conditioned media of cells stimulated with GPCR agonists (37). GPCRs employ multiple distinct pathways to activate the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) cascade. It has been reported that GPCR-stimulated tyrosine phosphorylation of EGFR involves the release of a soluble EGFR ligand, HB-EGF (38). HB-EGF is proteolytically cleaved at a juxtamembrane site, leading to the shedding of soluble HB-EGF, which activates EGFR in an autocrine/paracrine manner (17). We previously reported that shedding of EGFR ligands represents a critical event in keratinocyte migration and the early phases of wound healing (23).

Based on these observations, we examined how transactivation is involved in Ang II-induced keratinocyte and fibroblast migration. Our results confirm that Ang II induces EGFR phosphorylation in both keratinocytes and fibroblasts, with maximal activation occurring within 15 and 30 min, respectively. Ang II-induced cell migration was found to be inhibited by ErbB kinase inhibitor, HB-EGF neutralizing antibody, and...
CRM197, which indicates that Ang II-induced cell migration is mediated mainly by HB-EGF shedding through AT1R.

There have been several reports describing the role of metalloproteases in mediating EGFR transactivation and the subsequent functions induced by many GPCRs. Asakura et al. (39) reported that cellular signaling in cardiomyocytes following treatment with GPCR agonists is dependent upon EGFR transactivation triggered by ADAM 12-mediated cleavage of HB-EGF. Mifune et al. (40) concluded that Ang II stimulates ADAM 17-dependent HB-EGF shedding through AT1R in COS-7 cells. In this study, broad spectrum MMP inhibitor (GM6001) inhibited Ang II-induced migration, which suggests that shedding of EGF ligands was critical in these events. Given that different types of cells were used in these experiments, the metalloprotease responsible for HB-EGF shedding may depend on the particular cells and organs.

Our observation led to an interesting clinical question: is cutaneous wound healing delayed in individuals receiving AT1R antagonists to treat hypertension? Drug dosage information shows that the blood level of AT1R antagonist is lower than the dose used in our research. Takeda et al. (26) reported the effect of the AT1 blocker candesartan on re-epithelization and vascular growth in a rat model. The group receiving candesartan (10 mg/kg/day) showed delayed re-epithelization and dermal repair as well as a suppression of angiogenesis. However, in animals given 1 mg of candesartan/kg/day, angiogenesis was only slightly suppressed, and re-epithelization of the wounds was observed on day 12 after wounding. This result was almost the same as the control. A typical human dose of AT1R antagonist is ∼1 mg/kg/day, and thus wound healing should not be delayed in persons taking these drugs.

Takeda et al. (26) reported that bromodeoxyuridine incorporation into keratinocytes and myofibroblasts is enhanced via AT1R signaling and suppressed via AT2R signaling and that the recovery of keratinocytes and myofibroblasts in migration assays is accelerated by AT1R signaling but inhibited by AT2R signaling. Most of the known biological effects of Ang II are mediated through AT1R. We therefore examined Ang II receptor expression in keratinocytes and fibroblasts, which are the main cell types contributing to skin wound healing. AT1R was expressed in both human and mouse keratinocytes and fibroblasts, whereas AT2R was detected only in human and mouse fibroblasts. Recombinant Ang II enhanced the migration of keratinocytes and fibroblasts in vitro, while migration was inhibited by the AT1R-specific inhibitor valsartan. By contrast, neither keratinocyte nor fibroblast migration was inhibited by the AT2R blocker PD123319. These results indicated that Ang II-induced keratinocyte and fibroblast migration is mediated by AT1R.

Ang II has been shown to enhance growth factor production in fibroblasts, and several studies implicate Ang II in cardiac fibrosis because of stimulated fibroblast proliferation (41). However, these effects could result in part from the paracrine actions of growth factors from Ang II-stimulated fibroblasts. Indeed, the inhibition of fibroblast proliferation in cultures of neonatal cardiac cells reduces Ang II-induced protein synthesis in cardiomyocytes (42). A recent study demonstrated the importance of AT1R-expressing cardiomyocytes in the proliferative response of cardiac fibroblasts to Ang II (43). Ang II, via AT1R, also appears to up-regulate FGF-2 expression in cardiac myocytes (44). We found that Ang II-induced migration and EGFR activation are mediated via AT1R signaling in epidermal keratinocytes and dermal fibroblasts. Our result of delayed wound healing in AT1aR−/− mice is consistent with this observation.

The regulation of collagen synthesis is an important part of Ang II-induced wound healing (45, 46). Collagen homeostasis is regulated by a delicate dynamic balance of synthesis and degradation. The crucial role of collagen synthesis in dermal wound repair is well understood, and Ang II has been demonstrated to be closely associated with the production of extracellular matrix. In skin, fibroblasts are pivotal for collagen production, and numerous collagenous structures need to be reconstituted after injury. Recently we have reported that Ang II increased collagen production via AT1R but inhibited collagen production via AT2R in mouse neonatal skin fibroblasts and that AT1aR−/− fibroblasts showed decreased collagen production (47). The decreased collagen production as well as impaired cell migration in AT1aR−/− mice plays an important role in delayed wound healing in these mice. Moreover, AT1R activation led to enhancement of insulin-like growth factor-I-induced collagen synthesis (47). The regulation of collagen production by the antagonistic actions of AT1R and AT2R in skin fibroblasts also supports the pathophysiological significance of Ang II in the skin. A detailed analysis of the specific cell types expressing AT1R and AT2R and their localization in the process of skin wound healing is necessary for understanding the complete role of Ang II in cutaneous wound healing.

In conclusion, we have demonstrated for the first time that Ang II plays an important role in skin wound healing in vitro and in vivo and that it functions by accelerating both keratinocyte and fibroblast migration in a process mediated by HB-EGF shedding.

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