Adiponectin Regulates Cutaneous Wound Healing by Promoting Keratinocyte Proliferation and Migration via the ERK Signaling Pathway

Sayaka Shibata, Yayoi Tada, Yoshihide Asano, Carren S. Hau, Toyoaki Kato, Hidehisa Saeki, Toshimasa Yamauchi, Naoto Kubota, Takashi Kadowaki and Shinichi Sato

J Immunol published online 17 August 2012
http://www.jimmunol.org/content/early/2012/08/17/jimmunol.1101739

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/08/17/jimmunol.1101739.DC1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Adiponectin Regulates Cutaneous Wound Healing by Promoting Keratinocyte Proliferation and Migration via the ERK Signaling Pathway

Sayaka Shibata,* Yayoi Tada,* Yoshihide Asano,* Carren S. Hau,* Toyoaki Kato,* Hidehisa Saeki,* Toshimasa Yamauchi,‡ Naoto Kubota,† Takashi Kadowaki,† and Shinichi Sato*

Diabetic patients are at high risk of developing delayed cutaneous wound healing. Adiponectin plays a pivotal role in the pathogenesis of diabetes and is considered to be involved in various pathological conditions associated with diabetes; however, its role in wound repair is unknown. In this study, we elucidated the involvement of adiponectin in cutaneous wound healing in vitro and in vivo. Normal human keratinocytes expressed adiponectin receptors, and adiponectin enhanced proliferation and migration of keratinocytes in vitro. This proliferative and migratory effect of adiponectin was mediated via AdipoR1/AdipoR2 and the ERK signaling pathway. Consistent with in vitro results, wound closure was significantly delayed in adiponectin-deficient mice compared with wild-type mice, and more importantly, keratinocyte proliferation and migration during wound repair were also impaired in adiponectin-deficient mice. Furthermore, both systemic and topical administration of adiponectin ameliorated impaired wound healing in adiponectin-deficient and diabetic db/db mice, respectively. Collectively, these results indicate that adiponectin is a potent mediator in the regulation of cutaneous wound healing. We propose that upregulation of systemic and/or local adiponectin levels is a potential and very promising therapeutic approach for dealing with diabetic wounds. The Journal of Immunology, 2012, 189: 000–000.

A
dipose tissue, located beneath the skin, was initially recognized merely as a site of energy storage. However, increasing evidence indicates that adipose tissue secretes various bioactive molecules and exerts multiple functions in concert with the epidermis and dermis via endo-, para-, and autocrine pathways (1, 2). Adipose tissue is now considered to be a key participant in the development of skin pathophysiology. Bioactive molecules secreted from adipocytes are termed adipokines. Most adipokines, including TNF-α, IL-6, and resistin, increase the risk of metabolic syndrome (3). In contrast, another adipokine, adiponectin, ameliorates insulin resistance and mediates antidiabetic effects in liver and skeletal muscle (4, 5). Adiponectin-deficient mice exhibit insulin resistance and glucose intolerance. Administration of adiponectin increases glucose uptake and fat oxidation in muscle, reduces glucose production in liver, and improves insulin sensitivity (6). Thus, adiponectin is a crucial factor in the pathogenesis of diabetes. Adiponectin is present at relatively high concentrations 3–30 μg/ml in circulation, and adiponectin levels are lower in obese and type 2 diabetes patients (7). Accumulated evidence shows that adiponectin is involved in the various pathological conditions associated with diabetes, such as arterial sclerosis, nephropathy, collateral vessel development, and ocular complications (8–10).

Diabetic patients frequently suffer from severely impaired wound healing, with a lifetime risk of 15% for developing diabetic skin ulcers (11). Diabetic ulcers have a poor prognosis, and 15–27% of all diabetic ulcers lead to the surgical removal of bone (12). However, the mechanisms underlying impaired wound healing in diabetes are poorly understood (13). Wound healing involves a complex biological and molecular cascade of events, including inflammation, granulation tissue formation, re-epithelialization, and angiogenesis (14). Specifically, the re-epithelialization process is critical to optimal wound closure because of its role in wound contraction, which is mediated by keratinocyte proliferation and migration (14). These processes are regulated by the balance of various cytokines, chemokines, and growth factors released by adipose tissue as well as platelets, monocytes, and keratinocytes (1). Adipose tissue participates in cutaneous wound healing as a source of cytokines and growth factors, such as fibroblast growth factors (15), insulin-like growth factor (16), epidermal growth factor (EGF)-like growth factor (17), vascular endothelial growth factor, and angiopoietin-1 (18), and regulates skin wound repair in all layers of the skin via the endocrine/paracrine/autocrine pathways. Indeed, a recent study has shown that adipose tissue extracts, seeded over skin wounds, promotes wound repair (19). Furthermore, leptin, which is another representative adipokine and also secreted from adipocytes, induces keratinocyte proliferation and systemically and topically supplemented leptin improved re-epithelialization of wounds in leptin-deficient ob/ob mice and wild-type mice (20, 21).

In this study, to investigate the role of adiponectin during the cutaneous wound healing, we first focused on the functions of keratinocytes, key mediators of the re-epithelialization process, and
performed functional analyses regarding proliferative and migratory effects. We further examined how adiponectin would be involved in vivo during the wound repair process, using a mouse model of excisional skin wound healing in adiponectin-deficient and diabetic db/db mice. The results of this study indicate that adiponectin enhances proliferation and migration of keratinocytes through AdipoR1/AdipoR2 and the ERK signaling pathway in vitro and that adiponectin regulates the skin wound healing process in vivo by promoting keratinocyte proliferation and migration.

**Materials and Methods**

**Reagents**

Recombinant human adiponectin was isolated and purified as described previously (22). We verified that purified recombinant adiponectin was free of endotoxin using an endotoxin detection kit (GenScript, Piscataway, NJ). Abs against ERK, p-ERK, p38, p-p38, JNK, p-JNK, Akt, p-Akt, and β-actin were obtained from Cell Signaling Technology (Beverly, MA). Abs against adiponectin, AdipoR1, and AdipoR2 were from Abcam (Cambridge, U.K.). Abs against Ki67 and loricrin were obtained from Novo Castra Laboratories (Newcastle, U.K.) and Covance (Berkeley, CA), respectively. PD98059, U0126, wortmannin, LY294002, SB203580, and SP600125 were also purchased from Cell Signaling Technology.

**Cell culture**

Normal human epidermal keratinocytes were cultured with MCDB153 medium supplemented with insulin (5 μg/ml), hydrocortisone (1 μM), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine hypothalamic extract (BHE) (50 μg/ml), and Ca2+ (0.1 mM) as described previously (23, 24). Third or fourth passage cells were used in this study.

**RNA interference for adiponectin receptors**

All transfections were done using Lipofectamine 2000 following the manufacturer’s instructions (Invitrogen Life Technologies, Carlsbad, CA). Keratinocytes were plated in 6-well plates and cultured without antibiotics until reaching 30–50% subconfluency. AdipoR1/AdipoR2/calreticulin/T-cadherin and negative control small interfering RNA (siRNA) (Thermo Scientific Dharmacon, Lafayette, CO) were transfected into keratinocytes using Lipofectamine 2000. Medium was changed after 6 h after trans-
fection, and cells were incubated for another 24 h before subsequent analyses. Gene mRNA expression was examined by real-time RT-PCR analysis.

RT-PCR and quantitative real-time RT-PCR analysis

Total RNA was extracted from cultured keratinocytes and mouse back skin using an RNeasy Mini kit and RNeasy fibrous Tissue Mini kits, respectively (Qiagen, Germantown, MD), respectively. cDNA was synthesized using Superscript III First strand synthesis kits (Invitrogen).

For RT-PCR, synthesized cDNA was thermocycled for PCR amplification with 10 μM of each primer and 1.5 U Taq polymerase (Invitrogen). Primers for amplification, and the sizes of respective PCR products were as follows: AdipoR1, 5'-AATTCCTGACGCTTCTTCTT-3' and 5'-GATTAGGTCACCTCGAGTCTAAG-3' (25); calreticulin, 5'-AAGTGTCACGAGCAAGGAG-3' and 5'-GTCGAGTGCCTCAATGTCT-3' (26); c-adherin, 5'-GCCAGATGATGATGGTGATCGAGAC-3' and 5'-GGTCTCCATTTCCATTTGGA-3' (27); and glyceraldehyde-3-phosphate dehydrogenase gene, 5'-TGAAGGTCCGGAGTCAACGATTTGGT-3' and 5'-CATGTGGGCCATGAGGTCCACCAC-3' (28). PCR was performed with NuPAGE 4–12% Bis-Tris gels and MES running buffer (Invitrogen). After transfer to an Immobilon-P transfer membrane was performed with NuPAGE 4–12% Bis-Tris gels and stained with ethidium bromide, viewed by UV light.

For quantitative real-time RT-PCR (qRT-PCR), gene expression was quantified using TaqMan gene expression assay (Applied Biosystems, Warrington, U.K.). PCR conditions were 95˚C for 10 min, 40 cycles of 95˚C for 15 s, 60˚C for 45 s, and 72˚C for 45 s and a final extension at 72˚C for 3 min. The PCR products were analyzed by electrophoresis on 2.5% agarose gels and visualized by UV light. The relative change in the levels of genes of interest was determined by the 2 ΔΔCT method.

Western blot analysis

Subconfluent keratinocytes were lysed in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 μg/ml leupeptin. Samples were dissolved in NuPAGE LDS Sample Buffer with NuPAGE Sample Reducing Agent (Invitrogen) and denatured by heating 5 min at 95˚C. SDS-PAGE was performed with NuPAGE 4–12% Bis-Tris gels and MES running buffer (Invitrogen). After transfer to an Immobilon-P transfer membrane (Millipore, Bedford, MA), the membrane was incubated for 1 h at room temperature with blocking buffer, overnight at 4˚C with the primary Ab, washed, and incubated for 1 h at room temperature with the appropriate secondary Ab. After washing, visualization was performed by ECL (Amersham Biosciences, Buckinghamshire, U.K.). In some experiments, phospho-ERK1/2 levels were quantified by densitometric analysis using ImageJ (available online) and normalized to the total ERK 1/2 levels.

MTT assay

Keratinocytes were seeded on 12-well plates at a density of 2 × 10⁴ cells/well. The next day, the cells were fed MCDB153 medium lacking BHE, and the following day, they were fed once again with the same medium containing designated amounts of adiponectin. After 3 d, cell viability was assessed using an MTT assay kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer’s instructions.

BrdU incorporation assay

Keratinocytes were seeded on 96-well plates at a density of 1 × 10⁴ cells/well. After reaching subconfluence, the cells were fed MCDB153 medium lacking BHE. The following day, the cells were fed once again with the same medium containing designated amounts of adiponectin, EGF, and TGF-β and incubated for another 12 h. The cells were then incubated with medium containing BrdU for 2 h. BrdU incorporation was determined using a cell-proliferating ELISA kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer’s instructions.

Boyden chamber assay

Keratinocyte migration was also assayed quantitatively with a Boyden chamber, as described previously (29). Designated amounts of adiponectin and hepatocyte growth factor (HGF) were added to the bottom wells of a 48-well Boyden chamber (Neuro Probe, Gaithersburg, MD), and a 10-μm pore-size polyvinylpyrrolidone-free polycarbonate membrane (Neuro Probe) was placed on the wells. The membrane was precoated with type I collagen (10 μg/ml in PBS; Nitta Gelatin) at room temperature for 1 h and then washed extensively with PBS. Subconfluent keratinocytes were harvested with trypsin–EDTA (0.05% trypsin and 0.5 mM EDTA) and resuspended in culture medium without BHE at 1 × 10⁶ cells/ml. A 50-μl aliquot of the keratinocyte suspension (5000 cells/well) was added to the upper wells, and the chamber was incubated overnight at 37˚C in a humidified atmosphere of air with 5% CO₂. The cells that adhered to the upper surface of the filter membrane were removed by scraping with a rubber blade, and the cells that moved through the filter and stayed on the lower surface of the membrane were considered to be migrated cells. The membrane was fixed with 10% buffered formalin overnight and then stained with crystal violet. The membrane was then mounted between two glass slides with 90% glycerol, and the number of migrated cells was counted under a microscope.

In vitro wound closure assay

Keratinocytes were seeded on 6-well plates at a density of 1 × 10⁶ cells/well. After reaching subconfluence, wounds were created at the center of each well by scraping, and culture debris was removed by PBS washing. The remaining cells were cultured further with designated amounts of adiponectin and HGF. After 12 h, keratinocyte migration was observed under a phase contrast microscope. Wound closure was determined by identifying the front of cell migration and calculating a ratio of the migration area to the area of time 0.

Mice

Adiponectin-deficient mice were generated as described previously (6). BKS.Cg + Lepr db+ Lept db (db/db) were purchased from CLEA Japan (Tokyo, Japan). Mice were 7- to 10-wk-old for all experiments, and age-matched wild-type C57BL/6 mice (CLEA Japan) were used as controls for adiponectin-deficient and db/db mice. All mice were maintained under a 12-h light/12-h dark cycle in a specific pathogen-free barrier facility. All studies and procedures were approved by the Committee on Animal Experimentation of Tokyo University.

![FIGURE 2. Proliferative effects of adiponectin in keratinocytes. (A) After incubation for 3 d with indicated amounts of adiponectin, cell viability was assessed using the MTT assay. Data are shown as mean ± SE (n = 5) and are representative of four independent experiments. *p < 0.001 versus unstimulated keratinocytes. (B) After incubation for 12 h with indicated amounts of adiponectin, EGF (positive control), and TGF-β (negative control), BrdU incorporation was determined by ELISA. Data are shown as mean ± SE (n = 8) and are representative of four independent experiments. *p < 0.01, **p < 0.001 versus unstimulated keratinocytes.](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/)
Wounding and macroscopic examination

Mice were anesthetized with diethyl ether, and their backs were shaved and wiped with 70% alcohol. Four full-thickness excisional wounds per mouse were made using a disposable sterile 6-mm biopsy punch (Maruho, Osaka, Japan), and mice were caged individually. No signs suggestive of local infection were detected in the wounded skin. We excluded wounds with extreme distortion, which did not permit a precise determination of their size. Areas of open wounds were traced onto a transparency and analyzed using ImageJ (available online).

Histological assessment of wound healing

Skin sections of wounds including a 2-mm rim of unwounded skin tissue were taken from murine back skin. The samples were formalin fixed and embedded in paraffin. Five-micrometer sections were stained with H&E. The epithelial gap, which represents distance between the leading edge of migrating keratinocytes, was measured under a light microscope. For the analyses of staining for adiponectin, AdipoR1/AdipoR2, Ki67, and loricrin, immunohistochemistry was performed using a Vectastain avidin/biotin complex kit (Vector Laboratories, Burlingame, CA), according to the manufacturer’s instructions. The 5-μm sections were deparaffinized and rehydrated, endogenous peroxidase activity was eliminated by blocking with hydrogen peroxide, and the tissue sections were immersed in citrated buffer and boiled for 10 min for Ag retrieval. The sections were then incubated with indicated Abs overnight at 4°C, followed by the incubation with biotinylated secondary Ab. The concentration of each primary Ab was first tested to determine the optimal sensitivity range. The immunoreactivity was visualized with diaminobenzidine, and the sections were counterstained with Mayer’s hematoxylin.

Statistical analyses

Data obtained are presented as mean ± SE. Student t test was used for the statistical analysis of differences between two groups. One-way ANOVA with Dunnett’s multiple comparison test was used for statistical analysis of the differences among multiple groups. A p value < 0.05 was considered to represent a significant difference.

Results

Adiponectin receptors are expressed in human keratinocytes and AdipoR1 expression is upregulated by adiponectin stimulation

AdipoR1 is expressed widely in various tissues, with the highest expression level in skeletal muscle, whereas AdipoR2 is most abundantly expressed in the liver (30). Recently, new receptors for adiponectin, calreticulin, and T-cadherin also have been identified and reported to be involved in adiponectin cellular signaling (31–34). We first investigated the expressions of these adiponectin receptors in normal human keratinocytes. Human keratinocytes expressed both AdipoR1/AdipoR2 as well as calreticulin and T-cadherin at the mRNA level (Fig. 1A). Adiponectin upregulated gene expression levels of AdipoR1 in a dose-dependent manner but not those of AdipoR2, calreticulin, or T-cadherin in keratinocytes (Fig. 1B).

Adiponectin enhances keratinocyte proliferation

We next investigated the effect of adiponectin on keratinocyte proliferation using the MTT and BrdU assays. The MTT assay quantifies viable cells, whereas the BrdU assay determines BrdU incorporation into newly synthesized DNA of actively proliferating cells. Adiponectin increased the number of viable keratinocytes (Fig. 2A) and BrdU uptake (Fig. 2B) in a dose-dependent manner, reaching a plateau at a concentration of 25 μg/ml. Because plasma adiponectin concentrations in healthy humans are ∼3–30 μg/ml (36), the stimulatory effects of adiponectin were significant within physiological concentrations. EGF, a positive control, significantly induced keratinocyte proliferation in the BrdU assay, whereas TGF-β, a negative control, had no effect (37).

Adiponectin induces keratinocyte migration

After establishing that adiponectin stimulates keratinocyte proliferation, we next investigated the effect of adiponectin on keratinocyte migration using two types of in vitro assay systems. In the Boyden chamber assay, we quantitatively investigated adiponectin-induced migration. Various amounts of adiponectin and cultured human keratinocytes were added to the lower and upper chambers, respectively. After incubation overnight, the number of migrated cells was counted. Adiponectin significantly stimulated keratinocyte migration at concentrations of 12.5–50 μg/ml (Fig. 3A). HGF was used as a positive control, which induced an ∼7-fold increase relative to control (38).

FIGURE 3. Migratory effects of adiponectin in keratinocytes. (A) Keratinocyte migration was assayed quantitatively using the Boyden chamber assay. Indicated amounts of adiponectin and HGF were added to the lower wells. After keratinocytes were added to the upper wells and incubated overnight, the migrated cells were counted. Data are shown as mean ± SE (n = 6) and are representative of four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 versus unstimulated keratinocytes. (B) Keratinocyte migration was assayed using the in vitro wound closure assay. After subconfluent keratinocytes were scraped and incubated with indicated amounts of adiponectin and HGF for 12 h, migration over the wound was measured. Data are shown as mean ± SE (n = 6) and are representative of three independent experiments. *p < 0.01, **p < 0.001 versus unstimulated keratinocytes. (C) Representative micrograph of keratinocytes directly after (left panels) or 12 h after (right panels) scraping (original magnification ×20).
Next, wounds were created in cultured keratinocytes by scraping (in vitro wounds). Keratinocytes were cultured with various concentrations of adiponectin or HGF. Acceleration of wound closure was observed in response to 25 μg/ml adiponectin 12 h after wounding (Fig. 3B, 3C).

The ERK signaling pathway is activated by adiponectin via AdipoR1/AdipoR2 in keratinocytes

MAPK is a well-known factor involved in cell proliferation (39–41). Akt and AMP-activated protein kinase (AMPK) are also known to enhance cell proliferation and suppress apoptosis (10). Therefore, we investigated whether adiponectin could induce phosphorylation of MAPKs, Akt and AMPK in normal human keratinocytes. As shown in Fig. 4A, adiponectin (25 μg/ml) induced phosphorylation of ERK in keratinocytes. The peak activation occurred at 5 min after stimulation, and thereafter, the phosphorylation level gradually decreased. Adiponectin had no effect on the phosphorylation of the p38 MAPK, JNK, Akt, and AMPK (Fig. 4B). To confirm the specificity of the identified pathway, keratinocytes were pretreated with 75 μM MEK1 in-
hbitor (PD98059) and 10 μM MEK1/2 inhibitor (U0126) for 30 min and then stimulated with adiponectin for 5 min. Both of these inhibitors blocked adiponectin-induced ERK phosphorylation (Fig. 4C). To determine whether the activation of the ERK signaling pathway by adiponectin is mediated through AdipoR1/AdipoR2/calreticulin/T-cadherin, keratinocytes were transfected with siRNAs of these receptors, and specific inhibition was performed. Control experiments have revealed that AdipoR1/AdipoR2/calreticulin/T-cadherin mRNA levels are reduced by >80% as evaluated by real-time RT-PCR analysis (Fig. 4D).

Under these experimental conditions, inhibition of AdipoR1 or AdipoR2 completely suppressed adiponectin-induced ERK phosphorylation in keratinocytes (Fig. 4E, 4F). However, downregulation of calreticulin or T-cadherin did not significantly suppress adiponectin-induced ERK phosphorylation. These results indicate that 1) adiponectin activates the ERK signaling pathway through AdipoR1 and AdipoR2 and that 2) calreticulin or T-cadherin appear not to be involved in the ERK signaling pathway mediated by adiponectin in keratinocytes.

**ERK signaling pathway mediates adiponectin-induced keratinocyte proliferation and migration**

To investigate the role of the ERK signaling pathway in keratinocyte proliferation induced by adiponectin, we incubated keratinocytes with PD98059 (75 μM) or U0126 (10 μM) for 30 min before stimulation with adiponectin (25 μg/ml), and then, BrdU uptake was analyzed. Both of these inhibitors attenuated BrdU uptake induced by adiponectin, indicating that adiponectin-induced keratinocyte proliferation is mediated by the ERK pathway (Fig. 5A). Other kinase inhibitors (wortmannin and LY294002 for inhibition of PI3K, SB203580 for inhibition of p38 MAPK, and SP600125 for inhibition of JNK) did not affect adiponectin-induced proliferation. We further investigated whether the ERK signaling pathway would be involved in the keratinocyte migration induced by adiponectin. After the addition of PD98059 (75 μM) or U0126 (10 μM) to the lower chamber with adiponectin, keratinocyte migration was analyzed using the Boyden chamber assay. Both of the inhibitors blocked adiponectin-induced keratinocyte migration (Fig. 5B). These results suggest that the ERK signaling pathway mediated adiponectin-induced keratinocyte migration as well as proliferation.

**Wound closure and keratinocyte re-epithelialization are significantly delayed in adiponectin-deficient mice**

To further strengthen our in vitro results and to determine the role of adiponectin during cutaneous wound healing in vivo, we first examined the excisional wound skin repair process in adiponectin-deficient and wild-type mice. Full-thickness round wounds of 6 mm in diameter were made, and the kinetics of wound closure were evaluated as percentage of original wound areas. Adiponectin-deficient mice exhibited significantly impaired wound repair compared with wild-type mice from day 3 (Fig. 6A, 6B). All the wounds of wild-type mice healed within 9 d after wounding, whereas the wounds of adiponectin-deficient mice took more than 13 d to heal. Next, we focused on the re-epithelialization phase of wound healing, which involves keratinocyte proliferation and migration for wound contraction. To confirm the pattern of keratinocyte differentiation to be normal in the adiponectin-deficient mice, we first examined the expression of loricrin, a marker of late-phase keratinocyte differentiation, by immunohistochemistry. Loricrin expression in the keratinocytes was comparable between wild-type and adiponectin-deficient mice (Fig. 6C). Next, we performed immunohistochemistry for the proliferation marker Ki67 on the skin samples of the wound margins (20, 42). Distribution of Ki67-positive proliferating keratinocytes was more intensive in the wound margins of control wild-type mice compared with adiponectin knockout (KO) mice (Fig. 6D, 6E). Furthermore, the epithelial gap, the distance between the migrating edges of keratinocytes, was significantly wider in adiponectin-deficient mice compared with wild-type mice (Fig. 6A, 6F), suggesting that keratinocyte migration was significantly inhibited in adiponectin-deficient mice. These results are in accordance with the in vitro results regarding the proliferative and migratory functions of adiponectin in keratinocytes.

**The gene expression of adiponectin and AdipoR1/AdipoR2 gradually increases during the wound healing process**

To determine whether the local adiponectin and AdipoR1/AdipoR2 expression levels change during the wound healing process, we determined the changes in expression of these genes over time. Skin samples, taken immediately before (at day 0) and 3 and 6 d (days 3 and 6) after wounding, were analyzed by the qrt-PCR. We demonstrated in this study that adiponectin expression at the wound site gradually increased during the course of wound repair in wild-type mice (Fig. 7A), suggesting that adiponectin production might be upregulated sensing tissue damage of wounding. Immunohistochemistry of adiponectin showed that the sources of adiponectin are adipocytes and fibroblasts but not keratinocytes (Fig. 7B). Regarding adiponectin receptors, AdipoR1 and AdipoR2 mRNA expression also increased gradually after wounding in both wild-
type and adiponectin KO mice (Fig. 7C). The expression levels of these receptors during wound repair were comparable between wild-type and adiponectin-deficient mice. We also analyzed AdipoR1 and AdipoR2 expression on cells in wound samples of wild-type and adiponectin-deficient mice by immunohistochemistry. Protein expression of AdipoR1 and AdipoR2 was detected in fibroblasts and adipocytes as well as keratinocytes, and their expression was upregulated after wounding (Fig. 7D). Taken together, adiponectin, locally produced by adipocytes after skin damage, might induce the upregulation of AdipoR1 and AdipoR2 expression on keratinocytes. This process would further enhance adiponectin-mediated signaling within keratinocytes, thus promoting keratinocyte proliferation and migration.

Systemically supplemented adiponectin ameliorates delayed wound healing in adiponectin-deficient mice but not in wild-type mice

To determine whether the impaired wound healing in adiponectin-deficient mice is particularly adiponectin dependent or not, wild-type and adiponectin-deficient mice were given daily i.p. injection of recombinant adiponectin (50 μg/day/mouse) from 1 d before wounding, and the wound repair process was examined. As shown in Fig. 8A, systemic injection of physiological dose of adiponectin significantly ameliorated impaired wound healing in adiponectin-deficient mice but not in wild-type mice at days 3 and 7. These results may indicate that adiponectin supplementation is effective only under the condition when adiponectin is insufficient.

Topically supplemented adiponectin accelerates delayed wound healing in db/db mice but not in wild-type mice

To evaluate a potential clinical application of adiponectin, we investigated whether adiponectin would also act directly at the wound site by administering adiponectin to wound beds directly in db/db mice, a rodent model for type 2 diabetes, and wild-type mice. We applied 2.5 μg adiponectin in 50 μl PBS on the left side of the murine back and the same dose of PBS on the right side under the occlusive dressing every day. db/db mice were previously shown to have decreased adiponectin levels and impaired wound healing (43). Topical adiponectin administration significantly reduced open wound area relative to PBS administration at days 13, 16, and 19 (Fig. 8B) in db/db mice. Re-epithelialization was also assessed at day 13 microscopically, and the epithelial gap was significantly shorter in adiponectin-treated mice relative to PBS-treated mice (43). Topical adiponectin administration significantly reduced open wound area relative to PBS administration at days 13, 16, and 19 (Fig. 8B) in db/db mice. Re-epithelialization was also assessed at day 13 microscopically, and the epithelial gap was significantly shorter in adiponectin-treated mice relative to PBS-treated mice (43). Topical adiponectin administration significantly reduced open wound area relative to PBS administration at days 13, 16, and 19 (Fig. 8B) in db/db mice. Re-epithelialization was also assessed at day 13 microscopically, and the epithelial gap was significantly shorter in adiponectin-treated mice relative to PBS-treated mice (43).
Discussion

Adiponectin, a key mediator in the pathogenesis of diabetes, exerts multiple biological activities in various diseases; however, its role in cutaneous wound healing is unknown. To clarify this, we investigated pathophysiologic roles of adiponectin during cutaneous wound healing. We first demonstrated that keratinocytes express adiponectin receptors and that adiponectin induces proliferation and migration of keratinocytes in a dose-dependent manner through ERK activation in vitro. By using siRNAs of adiponectin receptors on keratinocytes, we also determined that AdipoR1 and AdipoR2 are mainly involved in the adiponectin-mediated ERK signaling pathway. These in vitro results suggest that adiponectin might contribute to the re-epithelialization phase of optimal wound healing. We have next undertaken a detailed functional analysis of adiponectin during wound repair in vivo. Wound closure was significantly delayed in adiponectin-deficient mice compared with wild-type mice. The number of Ki67-positive cells in the wound margin epithelial cells was decreased and re-epithelialization was delayed in adiponectin-deficient mice, which were supportive of in vitro results. The expression of AdipoR1/AdipoR2 as well as adiponectin gradually increased after wounding, which may consequently augment adiponectin signaling within keratinocytes and facilitate wound repair. Finally, we examined the curative effect of adiponectin on wounds. Both systemic and topical adiponectin supplementation normalized the impaired wound healing in adiponectin-deficient mice and/db/db mice, respectively. Thus, adiponectin might contribute to optimal cutaneous wound healing in diabetic patients, whose adiponectin levels are persistently decreased.

With regard to cell proliferation and migration, the behavior of adiponectin seems to depend on cell types. For instance, adiponectin stimulates cell growth in colonic epithelial cells (39), osteoblasts (40), and cardiac fibroblasts (41), where adiponectin is involved in the gastrointestinal mucosal metabolism, bone me-
Migratory effects of adiponectin have been reported in endothelial progenitor cells (44). In contrast, adiponectin suppressed cell proliferation and migration in vascular smooth muscle cells (45) and hepatic stellate cells (46), acting as a modulator for vascular remodeling and liver fibrosis, respectively. Most importantly, a certain subset of cell types that are involved in the cutaneous wound healing process exhibit proliferative and migratory behaviors in response to adiponectin. Dermal fibroblasts are the dominant players in the process of granulation tissue formation during cutaneous wound healing. Recent reports have revealed that adiponectin induces proliferation of dermal fibroblasts and upregulation of collagen production (47, 48). Furthermore, adiponectin induces proliferation of HUVECs and stimulates angiogenesis (10), indicating the positive contribution of adiponectin to optimal wound repair. In addition, adiponectin induces neutrophil migration, which is an initial step of skin wound repair (14). In this study, adiponectin exhibited proliferative and migratory effects on primary human keratinocytes. Proliferation and migration of keratinocytes are crucial factors of re-epithelialization, which marks the final stage of wound healing process. It should be mentioned that Kawai et al. (49) previously reported that adiponectin slightly suppressed the proliferation of the immortalized keratinocyte cell line HaCaT. Therefore, we also investigated whether adiponectin would exert effects on HaCaT cells as well; however, in our hands, adiponectin had no effect on proliferation of these cells (Supplemental Fig. 1). Although the reason for the discrepancy of the results between primary normal human keratinocytes and HaCaT cells is not clear, HaCaT cells have been reported to behave differently from normal human keratinocytes regarding cell growth, differentiation, and cytokine production in several reports (50, 51).

Consistent with our in vitro results, a significant impairment of wound repair was also observed in adiponectin-deficient mice in the current study. Furthermore, both systemic and topical adiponectin accelerated wound repair in adiponectin-deficient and diabetic db/db mice, respectively. In contrast, the wound healing rate of wild-type mice did not change after systemic or local administration of adiponectin. Thus, adiponectin has beneficial and direct effects on cutaneous wound healing, especially in diabetic patients whose adiponectin levels are constitutively decreased. These findings would suggest that upregulating serum adiponectin levels through medical treatment as well as raising cutaneous local adiponectin levels by direct administration of adiponectin to the wound sites represents a novel effective therapeutic target for delayed diabetic wounds (52).
JNK, ERK, PI3K, and Akt (10, 39–41). In the current study, we demonstrate that the proliferative and migratory activities of adiponectin are mediated through ERK signaling. It has been reported that the ERK signaling pathway is activated as a downstream signal molecule of adiponectin receptors in several cell types, including endothelial cells, macrophages, cardiac fibroblasts, and pancreatic cells (10, 41, 53, 54). Previous studies have shown that the ERK signaling pathway is activated upon injury or stretching of keratinocytes (55, 56). Interestingly, ERK pathways are required for wound healing in corneal epithelial cells under high glucose conditions (57). Considering that ERK activation is required for keratinocyte proliferation and migration (58), our present result showing that adiponectin activates the ERK signaling pathway in keratinocytes would suggest that adiponectin positively regulates the re-epithelialization process during cutaneous wound healing.

In summary, we propose a potent role for adiponectin, a key mediator of diabetes, in the regulation of keratinocyte proliferation and migration during cutaneous wound healing and provide a new mechanism underlying delayed wound repair in diabetic patients. On the basis of our data, we propose that systemic and/or local upregulation of adiponectin levels may represent a novel therapeutic strategy for diabetic wounds.

Acknowledgments
We thank Dr. Wolfgang W. Leitner (National Institute of Allergy and Infectious Diseases, National Institutes of Health) for helpful discussions.

Disclosures
The authors have no financial conflicts of interest.

References


