COMP-angiopoietin 1 Gene Transfer Enhances Cutaneous Wound Healing by Promoting Angiogenesis

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Background: Angiogenesis is crucial for wound healing and exogenous supplements of the angiogenic growth factors have been known to promote cutaneous wound healing. Angiopoietin (Ang) 1 is a recently discovered angiogenic factor and there have been few studies of its effect on cutaneous wound healing.

Objective: We examined the effect of Ang 1 on cutaneous wound healing.

Methods: Cartilage oligomeric matrix protein (COMP)-Ang 1 (Ade-COMP-Ang 1)- was intravenously injected to rats two days before surgery creating full-thickness wounds. The clinical wound healing rate and the number of vessels in the skin samples were evaluated on days 3, 7 and 14 post operation.

Results: At post-operation day 3, 7 and 14, the clinical wound healing rate was 38.3%, 59.4% and 92.1%, respectively, in the Ade-COMP-Ang 1-treated group, compared with 20.5%, 47.5% and 87.3%, respectively, in the Ade-LacZ-treated group. There were significant differences in the results of day 3 and day 7 between two groups ($p<0.05$). Histopathologically, the number of the vessels of the Ade-COMP-Ang 1-treated group was 73.7, 94.1 and 62.7 at day 3, 7 and 14, compared with that of the Ade-LacZ-treated group, 53.5, 83.9, and 56.9. The differences in the results of the two groups were statistically significant ($p<0.05$).

Conclusion: These results indicate that Ade-COMP-Ang 1 therapy significantly accelerats wound healing by promoting angiogenesis. However, further study using Ade-COMP-Ang 1 gene therapy for chronic wounds in which the formation of new blood vessels is impaired is needed in the near future.

INTRODUCTION

The closure of cutaneous wounds involves events such as hemorrhage, inflammation, re-epithelialization, granulation tissue formation, and the late remodeling phase of tissue repair. The early and acute phases of repair involve macrophage accumulation, fibroblast ingrowth, matrix deposition, and angiogenesis. These events are triggered by a complex mixture of cytokines and growth factors

that are released at the site of injury[1].

Angiogenesis is the sprouting of new blood vessels from a pre-existing network, and this process is central to the formation of granulation tissue because the ingrowth of newly formed vessels is needed to ensure the supply of oxygen, nutrients and inflammatory cells to the regenerating tissue[1,2]. Previous studies have shown that delayed wound healing such as diabetic ulcers, chronic venous ulcers, pressure ulcers, and cutaneous wounds in the aged, are associated with decreased angiogenesis and altered levels of angiogenic growth factors including vascular endothelial growth factor (VEGF) and angiopoietin (Ang)[3-5]. Based on these results, "therapeutic angiogenesis", i.e. supplementation of such recombinant angiogenic growth factors as VEGF, erythropoietin, nerve growth factor, platelet-derived growth factor, fibroblast growth factor[2] and granulocyte-machrophage colony stimulating factor, has been used both in animal models and in clinical trials for patients to enhance cutaneous wound healing[6-8].

Ang was discovered as a ligands for the tyrosine kinase with immunoglobulin and epidermal growth factor homology (Tie) 2 that was selectively expressed on the vascular endothelium. There are four definitive members of the Ang family, Ang 1, 2, 3 and 4. Although the functions of Ang and Tie have not been well established, Ang is thought to be involved in the proliferation, maturation, stabilization and remodeling of vessels[1,9]. Therefore, Ang 1 could be one of the promising candidate growth factors for therapeutic angiogenesis.

Large-scale production of recombinant Ang 1 is hindered by the aggregation and insolubility of protein. The activity of the purified protein frequently varies. These difficulties are due to its unique structural characteristics. COMP-Ang 1 is a soluble, stable, and potent Ang 1 variant in which the N-terminal portion of Ang 1 is replaced with the short coiled-coil domain of cartilage oligometric matrix protein (COMP). COMP-Ang 1 is more potent than native Ang 1 in phosphorylating Tie 2 and Akt in primary cultured endothelial cells and in angiogenesis in vivo[10,11].

In this study, we investigated the potential benefits of the therapy with adeno-associated systemic COMP-Ang 1 on cutaneous wound healing. The results of the study show that systemic COMP-Ang 1 treatment enhances cutaneous wound healing in vivo by promoting angiogenesis.

MATERIALS AND METHODS

Adenoviral vectors

Recombinant adenoviruses that expressed COMP-Ang 1 or LacZ were constructed using the pAdEasy vector system (Ade-COMP-Ang 1 and Ade-LacZ) (Ade-COMP-Ang 1 and Ade-LacZ were generous gifts from Prof. Gou Young Koh at Korea Advanced Institute of Science and Technology, Daejeon, Korea).

Animals and adeno viral treatment

Eighteen male Sprague-Dawley rats (200~250 g in weight) were housed two per cage, maintained under controlled environmental conditions (12 h light to dark cycle, temperature approximately 23°C) and provided with standard laboratory food and water ad libitum. All the animal procedures were in accordance with the declaration of Helsinki and the guidelines for the care and use of laboratory animals. The animal care and experimental procedures were performed under approval from the Animal Care Committees of the Catholic University of Korea. For the adeno-viral treatment, 10^9 p.f.u. of Ade-COMP-Ang 1 and Ade-LacZ diluted in 50 μL of sterile 0.9% NaCl was injected intravenously into nine rats, through the tail vein two days before surgery.

Animal wound model

General anesthesia with ketamine (80 mg/kg body weight)/xylazine (10 mg/kg body weight) was achieved by intraperitoneal injection. The hair on the back of each rat was shaved and the back skin was subsequently wiped with 70% ethanol. Three full-thickness wounds (1 cm in diameter, 2~3 cm apart to minimize the tension to adjacent wounds) were made on the back of each rat by excising the skin. The wounds were allowed to form scabs.

Determination of wound healing rate

To compare the rate of wound healing between the Ade-COMP-Ang 1- and Ade-LacZ-treated groups, the animals were photographed on days 0, 3, 7 and 14 after surgery. In addition, the wounds were copied exactly onto transparent film with an indicated standard unit. The photographs and

transparent films were digitally processed and the areas of the wounds were calculated using the KS300 system (Zeiss, Jena, Germany). For each sample, the rate of the healing process was measured as a percentage of the area of the wound at each time-point compared to the area of the wound at day 0.

Histology and immunohistochemistry

Three wounds on the back of each rat were sequentially removed from the site at days 3, 7 and 14 after surgery. Each specimen was divided into two equal parts. One half of the specimens were fixed in 4% paraformaldehyde, washed in tap water, dehydrated in a series of graded ethanol solutions, cleared in xylene, and then embedded in paraffin for light microscopic examination. The sections (4 μm thickness) were then mounted on glass slides, hydrated with distilled water, and subjected to hematoxylin-eosin staining (Vector Laboratories, Burlingame, CA, USA)[6].

The other half of each specimen was embedded in OCT compound (Sakura, Tokyo, Japan) for immunostaining. The skin specimens were prepared for immunohistochemical staining as previously described[6]. Briefly, five-micrometer cryostat sections were cut on a cryostat microtome (Leica Microsystems AG, Wetzlar, Germany), and then fixed in acetone for 15 min. After three washes in phosphate buffered saline (PBS), the sections were incubated for 10 minutes in methanol with 0.3% hydrogen peroxide to block the endogenous peroxidase activity. Nonspecific antibody binding was blocked by incubating the sections with 10% normal donkey serum (Jackson Immuno Research Laboratories, West Grove, PA, USA) for 60 min. The sections were incubated with mouse anti-rat PECAM-1/CD31 monoclonal antibodies (1 : 100 in PBS, Cemicon, CA, USA) overnight at 4°C. Subsequently, the tissue sections were incubated with secondary antibodies (1 : 300 in PBS, Jackson Immuno Research Laboratories) for 60 min. As controls, the same skin specimens were incubated with an isotype-matched antibody. As a negative control, skin samples were incubated with PBS without the primary antibodies. The color reaction of the treated tissue was carried out using the substrate 3-amino-9-ethylcarbazole (Vector Laboratories) for 10 min. The skin sections were also counterstained with hematoxylin (Vector Labora-

tories) for 1 to 2 min.

The number of blood vessels with definite lumens or with red blood cells in it was counted under a light microscope under the high power view (×200) by two independent dermatologists who were without knowledge of the previous treatment, and then the mean number and standard deviation of vessels in the Ade-COMP-Ang 1-treated and Ade-LacZ-treated groups were calculated.

Statistical analysis

The data were analyzed by two-way repeated measures analysis of variance (ANOVA) tests, two samples t-tests and paired t-tests were performed for statistical analysis. The data were analyzed by the statistical program SPSS for Windows 10.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as $p < 0.05$.

RESULTS

Ade-COMP-Ang 1 enhances the rate of wound healing

Analysis of the clinical wound healing rate in Ade-COMP-Ang 1-treated and Ade-LacZ-treated groups was performed via digital processing at 0, 3, 7 and 14 days after the wounding. The Ade-COMP-Ang 1-treated group showed 39.5%, 59.3%, and 91.8% reduction of the wound area at day 3, 7, and 14, respectively. On the other hand, the results in the Ade-LacZ-treated group revealed 20.4%, 46.2% and 89.0% reduction of the wound area, respectively. The differences in the results between the two groups at day 3 and 7 were statistically significant ($p < 0.05$), however, the differences in the results at day 14 failed to show a statistical significance (Fig. 1).

Ade-COMP-Ang 1 promotes the formation of new vessels

In order to investigate whether the Ade-COMP-Ang 1-induced-acceleration of wound healing was associated with an angiogenic effect, the number of vessels of the samples was evaluated by two independent investigators under a high power view (×200) at 3, 7, and 14 days after wound surgery. The mean vessel counts per specimen were averaged to give an overall mean vessel count per high-power field for each wound section. The
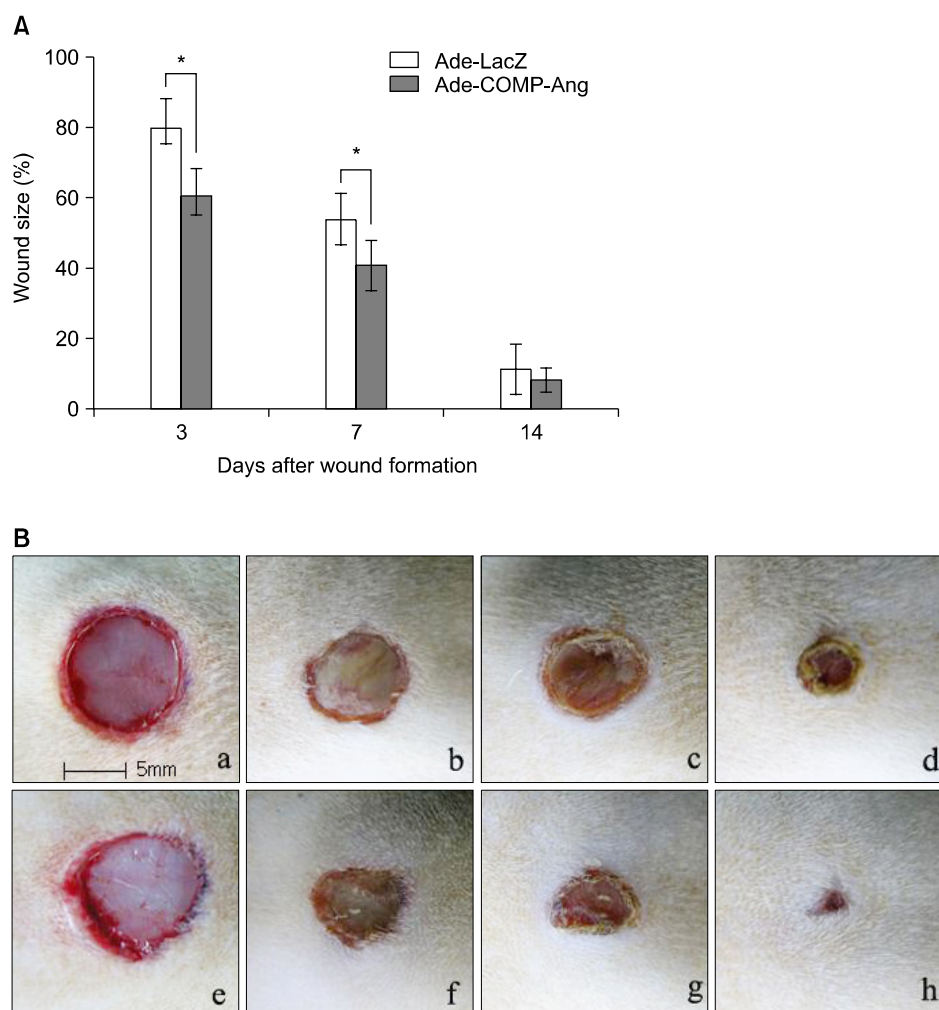
Fig. 1. (A) The effect of Ade-COMP-Ang 1 on clinical wound healing. The treatment of Ade-COMP-Ang 1 showed 39.5%, 59.3% and 91.8% reduction of wound size at day 3, 7 and 14, compared with 20.4%, 46.2% and 89.0%, in the Ade-LacZ treated group, respectively. The results of day 3 and 7 between the Ade-COMP-Ang 1 treated group and the Ade-LacZ treated group were statistically significant (*$p < 0.05$). (B) A representative example of the cutaneous wound healing of the Ade-COMP-Ang 1-treated and Ade-LacZ treated groups at day 0, 3, 7 and 14. The wound healing rate was accelerated in the Ade-COMP-Ang 1-treated group, compared with Ade-LacZ-treated group. Ade-LacZ-treated group: a (day 0), b (day 3), c (day 7), d (day 14 after wound surgery), Ade-COMP-Ang 1-treated group: e (day 0), f (day 3), g (day 7), h (day 14 after wound surgery).

number of blood vessels of the Ade-COMP-Ang 1-treated group was 73.7, 94.1, and 62.7 at day 3, 7, and 14, compared with the Ade-LacZ-treated group, i.e. those number of blood vessels was 53.5, 83.9 and 56.9, respectively. There were significant differences in the results at day 3, 7 and 14 between the two groups ($p < 0.05$) (Fig. 2).

DISCUSSION

The angiogenic effect of Ang 1 has been reported in previous studies. Ang 1 and Tie 2-deficient mice had similar phenotypes that were characterized by embryonic lethality with severe vascular remodeling defects, insufficient vessel stabilization and per-
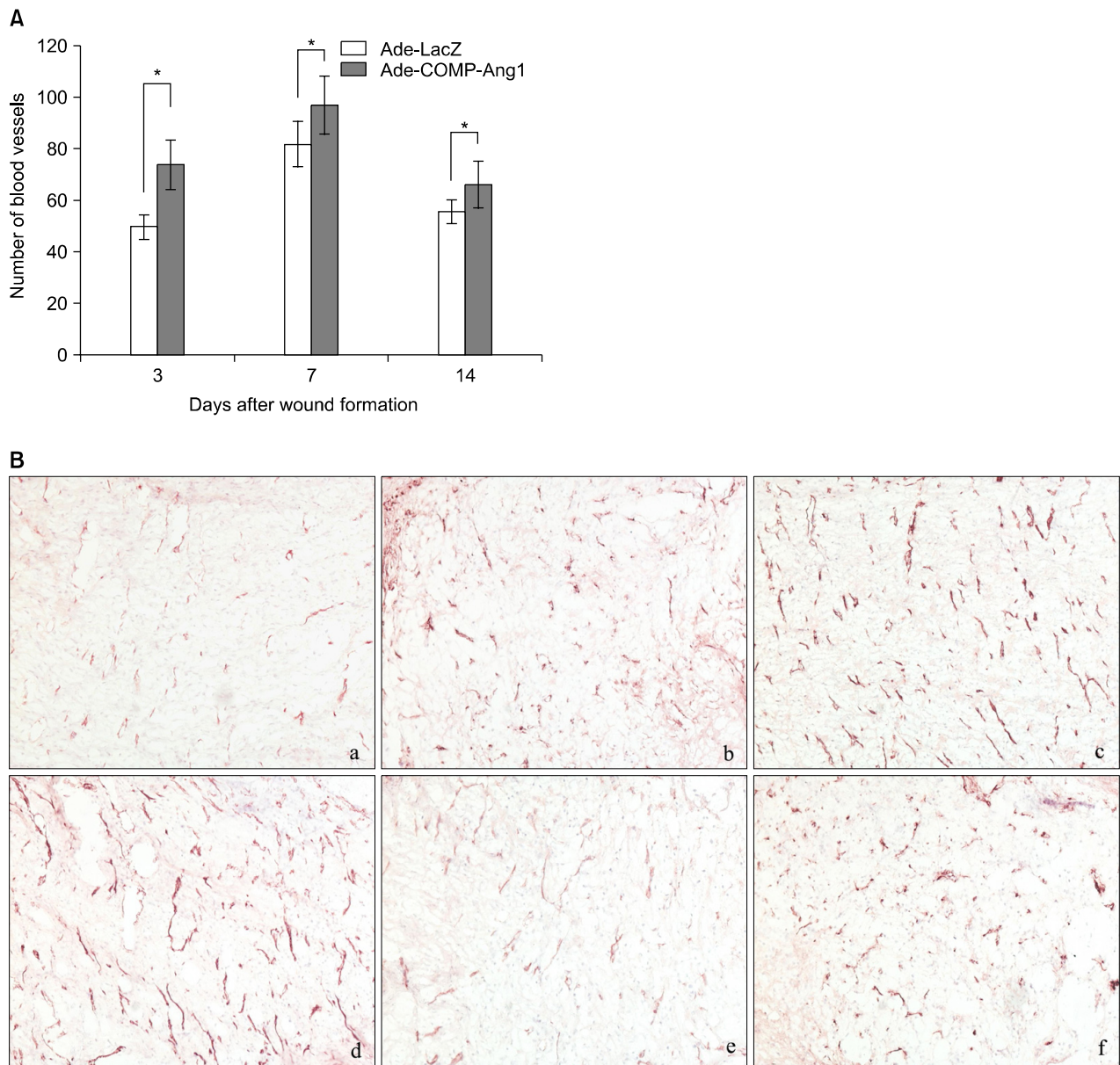
Fig. 2. (A) The effect of Ade-COMP-Ang 1 on angiogenesis. Under the high power field view (×200), the number of blood vessels of the Ade-COMP-Ang 1-treated group was 73.7, 94.1 and 62.7 at day 3, 7 and 14, respectively, compared with that of the Ade-LacZ treated group, i.e., 53.5, 83.9, and 56.9, respectively. The results of day 3, 7 and 14 between the Ade-COMP-Ang 1 treated group and the Ade-LacZ treated group were statistically significant (*$p < 0.05$). (B) The blood vessel formation of the Ade-COMP-Ang 1 treated and Ade-LacZ treated groups at day 3, 7 and 14 (PECAM, ×200). The number of blood vessels were increased in the the Ade-COMP-Ang 1-treated group, compared with Ade-LacZ-treated group. Ade-LacZ-treated group: a (day 3), c (day 7), e (day 14 after wound surgery), Ade-COMP-Ang 1-treated group: b (day 3), d (day 7), f (day 14 after wound surgery).

turbed vascular maturation[12,13]. Promoted angiogenesis has been reported on for transgenic mice that overexpress Ang 1 in the skin[14]. In addition, there have been reports that exogenous and localized Ang 1 treatment with naked DNA-mediated or adenovirus-mediated Ang 1 enhanced angiogenesis in the gastric ulcer model or the skin flap model[15,16]. In this study, we wanted to verify whether a sys-

temic adenovirus-associated COMP-Ang 1 treatment can induce angiogenesis in a cutaneous wound healing model. Our results demonstrated that angiogenesis in the COMP-Ang 1 treated group was significantly increased at day 3, 7 and 14, compared with the control group. Although we did not confirm the expression of COMP-Ang 1 either in the plasma or in the cutaneous wound after systemic injection, the level of systemic circulating COMP-Ang 1 is known to be increased as early as 12 hours after Ade-COMP-Ang 1 injection, peaking at 1 to 2 weeks and gradually declining thereafter. The levels return to the control levels at 6 weeks after systemic treatment in the murine model[10]. Therefore, we could infer that the enhanced angiogenesis in the COMP-Ang 1 treated group is attributable to the increased level of COMP-Ang 1 in the plasma.

The results of this study showed that adenovirus mediated COMP-Ang 1 therapy significantly enhanced the reduction of wound size at day 3 and 7 after wound surgery; however, there was no significant difference at day 14. These data coincided with previous reports showing the difference of the reduction of the wound size did not reach statistical significance after day 7 or 9 after wounding, in an adenovirus-mediated VEGF treatment model or a cultured autologous fibroblast transplant model, respectively[6,17].

A recent explosion of newly discovered growth factors that act on the vascular endothelium has coincided with the application of powerful new genetic approaches to the problem of vascular development. The vascular endothelium-specific growth factors include five members of the VEGF family, four members of the Ang family, and at least one member of the large ephrin family[1]. In addition, many other growth factors that are not vascular endothelium-specific are also required for blood vessel formation, and these include the members of the platelet-derived growth factor families or the transforming growth factor families. However, the critical roles on many other systems of the latter limit their clinical application. VEGF has been the most commonly studied and used growth factor for promoting wound healing. The previous studies have shown that VEGF is a more potent growth factor for endothelial proliferation than is Ang 1[15]. However, continual overexpression of VEGF has been found to result in hemangioma-type tumors[18],

indicating that the VEGF expression or effective dose must be tightly regulated and that the expression must transiently occur only at the early stage of wound healing. Furthermore, the microvessels that develop from the continual overexpression of VEGF were found to be disorganized, sinusoidal and leaky because of VEFG's additional function as a vascular permeability factor[19]. In contrast, Ang 1 induces maturation, stabilization and remodeling of vessels and overexpression of Ang 1 does not result in vascular leakage[20-22]. Therefore, Ang 1 is thought to have a wider therapeutic window and it could be a safer modality for clinical use.

As far as treatment with growth factors is concerned, naive protein treatment in the clinical setting is limited by several factors, such as their short half-lives, their inactivation by wound proteases, their poor bioavailability from the utilized vehicles and consequently the need for daily applications and high initial doses that might become toxic[23]. An alternative approach that might overcome most of these problems is the delivery of growth factor-encoding genes. Gene therapy could overcome the short-comings of direct application of the growth factor and so promote continual production and release of the growth factor within the blood.

In conclusion, the results of this study suggest that Ade-COMP-Ang 1 gene therapy significantly accelerates acute cutaneous wound healing by promoting angiogenesis at the site of injury. Further study using Ade-COMP-Ang 1 gene therapy in chronic wounds will be needed in the near future.

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