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Full Paper

Acemannan Stimulates Gingival Fibroblast Proliferation; Expressions of Keratinocyte Growth Factor-1, Vascular Endothelial Growth Factor, and Type I Collagen; and Wound Healing

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Abstract. *Aloe vera* has long been used as a traditional medicine for inducing wound healing. Gingival fibroblasts (GFs) play an important role in oral wound healing. In this study, we investigated the effects of acemannan, a polysaccharide extracted from *Aloe vera* gel, on GF proliferation; keratinocyte growth factor-1 (KGF-1), vascular endothelial growth factor (VEGF), and type I collagen production; and oral wound healing in rats. [^{3}H]-Thymidine incorporation assay and ELISA were used. Punch biopsy wounds were created at the hard palate of male Sprague Dawley rats. All treatments (normal saline; 0.1% triamcinolone acetonide; plain 1% Carbopol®; and Carbopol® containing 0.5%, 1%, and 2% acemannan (w/w)) were applied daily. Wounded areas and histological features were observed at day 7 after treatment. From our studies, acemannan at concentrations of 2, 4, 8, and 16 mg/ml significantly induced cell proliferation (P<0.05). Acemannan concentrations between 2 – 16 mg/ml significantly stimulated KGF-1, VEGF, and type I collagen expressions (P<0.05). Wound healing of animals receiving Carbopol® containing 0.5% acemannan (w/w) was significantly better than that of the other groups (P<0.05). These findings suggest that acemannan plays a significant role in the oral wound healing process via the induction of fibroblast proliferation and stimulation of KGF-1, VEGF, and type I collagen expressions.

Keywords: acemannan, *Aloe vera*, proliferation, growth factor, oral wound healing

Introduction

Ulceration is common lesion in the oral cavity. It causes discomfort and pain, especially in patients who have systemic conditions. Wound healing is a complex process that involves many different cell types in migration, proliferation, differentiation, removal of damaged tissue, and formation of extracellular matrix (1). Generally, wound healing can be divided into three overlapping phases: first, inflammation; second, proliferation and extracellular matrix synthesis; and third, remodeling (1). Gingival fibroblasts (GFs) play important roles in the proliferative phase of oral wound healing (2). They proliferate and secrete several growth factors and extracellular matrix, such as keratinocyte growth factor-1 (KGF-1), vascular endothelial growth factor (VEGF), and type I collagen, to generate new tissue (2).

Because of the possible adverse effects from synthetic chemicals, the trend is shifting toward the use of herbal medicine (3). Several herbal and natural products have been investigated for the promotion of wound healing (4, 5). *Aloe vera* (*Aloe barbadensis* MILLER) has long been used as an herbal medicine for healing skin wounds (6). It has been reported that *Aloe vera* gel stimulated dermal wound healing in rats by increasing collagen and glycosaminoglycan synthesis (7, 8). In any event, the active ingredient of *Aloe vera* gel has never been

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identified. Acemannan, β-(1,4)-acetylated polymannose, is the major polysaccharide of *Aloe vera* gel (9). Reports have stated that it exerts an immunostimulative effect by activating macrophages (10, 11). However, the roles of acemannan on GF proliferation and in vivo oral wound healing have never been reported.

The objectives of this study were to investigate the effects of acemannan on GF proliferation; KGF-1, VEGF, and type I collagen expressions; and oral wound healing in rats. The data obtained from this study will explain possible mechanisms of acemannan and *Aloe vera* gel on oral wound healing.

Materials and Methods

Preparation, purification, and characterization of acemannan

Aloe barbadensis MILLER (*Aloe vera* LINN.) was obtained from a local herbal supplier in Jatujak market (Thailand), and the specimen (AC-05-2007) was deposited at the Oral Biology Research Center, Faculty of Dentistry, Chulalongkorn University (Bangkok, Thailand). *Aloe vera* was identified by Assoc. Prof. Dr. Chaiyo Chaichantipyuth, Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. Acemannan was extracted from fresh *Aloe vera* pulp gel by homogenization, centrifugation, and alcohol precipitation as previously described (12). The molecular weight was analyzed by using high performance liquid chromatography (HPLC; Shimadzu, Kyoto) connected to a reflective index detector (RID-10A, Shimadzu). The separation was performed with a Shodex Sugar KS-804 column (8×300 mm; Showa Denko K.K., Kanagawa) comparison with Shodex standard P-82 (Showa Denko K.K.). The monosaccharide compositions was analyzed by using gas chromatography and mass spectroscopy (GC-MS) and ^{13}C-NMR spectroscopy as previously described (13, 14). The data obtained from these analyses were consistent with previous results and outcomes from T.-N. Chow et al., (13, 14) that revealed that the polysaccharide extracted from fresh *Aloe vera* gel is acemannan. The yield of acemannan extraction was about 0.2%.

Cell culture

Human GFs were prepared from explanted culture of gingiva obtained from surgical removal of impactions, under a protocol approved by the Ethics Committee, Faculty of Dentistry, Chulalongkorn University, Thailand. GFs were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; HyClone®, Logan, UT, USA); 10,000 IU/ml penicillin G sodium, 100,000 μg/ml streptomycin sulfate, 25 μg/ml amphotericin B (all from GIBCO™, Grand Island, NY, USA); and 1% L-glutamine (HyClone®) at 37°C, under 5%-CO_2 condition. All experiments were performed using cells from the third to the fifth passage.

DNA synthesis assay

DNA synthesis was investigated by [^{3}H]-thymidine incorporation assay. The assay was performed according to Yoshizumi et al. (15) with minor modifications. Briefly, GFs (6×10^4 cells/well) were grown in 24-well tissue culture plates in DMEM containing 10% FBS until the cultures were 70% – 80% confluent. Then cells were changed to serum-free medium for 3 h, twice. After that, cells were treated with different concentrations of acemannan. Control cultures received the same volume of culture medium without acemannan. Cells were labeled with 0.25 μCi/well of [^{3}H]-thymidine (Amersham Biosciences, Little Chalfont, UK) during the last 6 h of the incubation period. After 24 h, the medium was gently aspirated from each well. The cell layers were washed 3 times with PBS and fixed with 10% TCA for 20 min. Then the cell layers were washed again twice with 5% TCA and solubilized in 0.5 M NaOH overnight. After that, the sample in each well was neutralized with 0.5 M HCl, and then transferred to a scintillation vial. Scintillation fluid (OptiPhase HiSafe; Wallac, Milton Keynes, UK) was added to the vials and then mixed thoroughly. The amounts of beta radiation from [^{3}H]-thymidine were counted by a liquid scintillation counter (Wallac, Turku, Finland). The assay was carried out in three independent experiments.

Measurement of KGF-1, VEGF, and type I collagen production

The amounts of KGF-1, VEGF, and type I collagen were determined by enzyme-linked immunosorbent assay (ELISA) kits (R&D System, Minneapolis, MN, USA, for KGF-1 and VEGF; Takara, Shiga, for procollagen type I) according to the manufacturers' instructions. Briefly, GFs (7×10^4 cells/well) were grown to confluence in 24-well tissue culture plates. Cells were changed to serum-free medium for 3 h, twice. Then the medium was replaced by various concentrations of acemannan. A medium without acemannan was included as a control. After 24 and 48 h of incubation, culture supernatant was collected and stored at –20°C until used. According to the manufacturers' descriptions, the sensitivity of ELISA kits is less than 15 pg/ml, 5 pg/ml, and 10 ng/ml for KGF-1, VEGF, and procollagen type I, respectively. The assay was carried out in three independent experiments.

In vivo oral wound healing assay

The protocol was approved by the Animal Ethics Committee, Faculty of Veterinary Science, Chulalongkorn University, Thailand. The procedures were performed according to Oda et al. (16) with minor modifications. Sixty male Sprague Dawley rats (8-week-old, 200 – 250 g; National Laboratory Animal Center, Thailand) were used to evaluate the effect of acemannan on oral wound healing. The animals were kept on a 12-h light / 12-h dark cycle, fed a standard pelleted diet, and allowed water and diet ad libitum. The animals were anesthetized with an intraperitoneal injection of 80 mg/kg Zoletil® (chloral hydrate tiletamine and chloral hydrate zolazepam; Virbac Laboratories, Carros cedex, France). A 4-mm mucosal defect was made on the midline of hard palate to the depth of the periosteum using a punch biopsy (Kruuse®; Marsley, Denmark). Animals were divided equally into 6 groups. In group I, animals were topically treated with normal saline as a negative control. Those in group II were treated with triamcinolone acetonide 0.1% in dental paste (Kenalog®; Bristol-Myers Squibb, Princeton, NY, USA) as a reference treatment. In group III, animals were treated with plain Carbopol® 934P NF (Noveon™, Cleveland, OH, USA) mucoadhesive polymer. In groups IV, V, and VI, animals received Carbopol® 934P NF containing 0.5%, 1%, and 2% acemannan (w/w), respectively. All vehicles were applied daily. At days 3 and 7 after treatment, five animals in each group were sacrificed and wound areas were measured by using Image-Pro® Plus software version 4.5 (Media Cybernetics, Bethesda, MD, USA). The tissues around the wound areas were dissected and fixed with 10% formalin, embedded in paraffin, and then sectioned at 5-μm thickness. The tissues were stained with hematoxylin and eosin and then photographed at ×40 to ×400 magnifications under a light microscope (Olympus, Tokyo).

Statistical analyses

For the in vitro study and wound areas of rats, the results were each expressed as a mean ± S.E.M. The results were analyzed by one-way analysis of variance (ANOVA) using the SPSS program for Windows, version 11.5 (SPSS, Inc., Chicago, IL, USA). A Duncan multiple comparison test was used for post-hoc analysis. Values of $P<0.05$ were considered as statistically significant. The histological features of the rats' wounds were described.

Results

Acemannan induced GF proliferation

After incubating cells for 24 h, acemannan at concentrations of 2, 4, 8, and 16 mg/ml significantly induced newly DNA synthesis ($P<0.05$), as shown in Fig. 1. The maximum effect of acemannan was observed at a concentration of 8 mg/ml, which stimulated de novo DNA synthesis to a level approximately 9.63 times that of the untreated group. In addition, acemannan showed a dose-dependent effect on GF proliferation at concentrations between 0.25 – 8 mg/ml. Interestingly, all of the acemannan concentrations used in the present study had no cellular toxicity towards GFs.

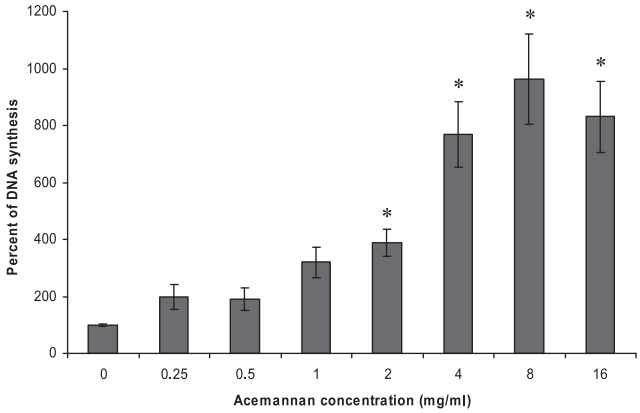

Fig. 1. Acemannan-induced GF proliferation. GF proliferation was examined by incorporation of [^{3}H]-thymidine after 24-h incubation with various concentrations of acemannan. Acemannan stimulated GF proliferation at concentrations of 2, 4, 8, and 16 mg/ml. *Compared to the untreated group; $P<0.05$, n = 3.

Acemannan stimulated KGF-1, VEGF, and type I collagen production

KGF-1, VEGF, and type I collagen secretions were evaluated by incubating GFs in the presence of various acemannan concentrations. The culture supernatants were collected at 24 and 48 h for quantification of protein level. At 24 h, ELISA revealed that acemannan at concentrations of 2, 4, 8, and 16 mg/ml significantly stimulated KGF-1 secretions at 24 h (Fig. 2A, white bar, $P<0.05$). In addition, acemannan at concentrations of 8 and 16 mg/ml significantly induced VEGF secretions (Fig. 2B, white bar, $P<0.05$). Acemannan at concentrations of 4, 8, and 16 mg/ml also significantly enhanced type I collagen synthesis at 24 h (Fig. 2C, white bar, $P<0.05$).

After 48 h of exposure, the enhancing effects of acemannan on KGF-1, VEGF, and type I collagen production were maintained. Acemannan at concentrations of 2 and 4 mg/ml produced continuously significant stimulation of KGF-1 expression (Fig. 2A, dark bar, $P<0.05$). Acemannan at concentrations of 4, 8, and 16 mg/ml significantly enhanced VEGF secretion from GFs (Fig. 2B, dark bar, $P<0.05$). Additionally, aceman-

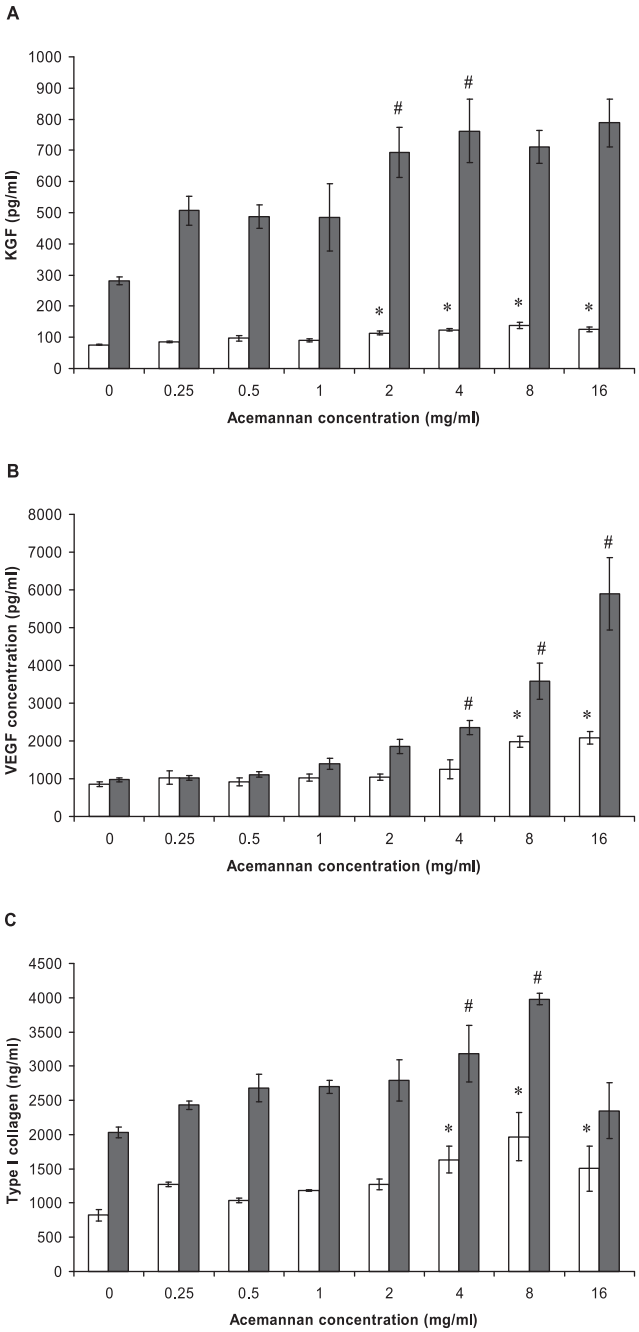

Fig. 2. KGF-1, VEGF, and type I collagen expressions from GFs in response to acemannan. KGF-1 (A), VEGF (B), and type I collagen (C) productions were determined by ELISA of GF culture supernatants after 24-h (white bar) and 48-h (dark bar) incubation with various concentrations of acemannan. Acemannan significantly enhanced production of KGF-1, VEGF, and type I collagen. *Compared to the untreated group at 24 h; #Compared to untreated group at 48 h; each at $P<0.05$, n = 3.

nan at concentrations of 4 and 8 mg/ml had a significant stimulative effect on type I collagen synthesis (Fig. 2C, dark bar, $P<0.05$).

Acemannan accelerated oral wound healing

At day 3 after treatment, there was no statistically significant difference in the reduction of wound areas in each treatment. However, acemannan-treated groups had slightly smaller wound areas than that of normal saline, plain Carbopol®, and triamcinolone acetonide (TA). At day 7 after treatment, TA; plain Carbopol®; and Carbopol® containing 0.5%, 1%, and 2% acemannan had a significant effect on the reduction of wound area as compared with normal saline, the negative control ($P<0.05$). The wound size of animals that received TA; plain Carbopol®; Carbopol® containing 0.5%, 1%, and 2% acemannan; and normal saline were 7.12 ± 0.36; 11.05 ± 1.44; 4.63 ± 0.73, 7.24 ± 0.47, and 6.77 ± 0.58; and 16.27 ± 3.04 mm^2, respectively. There was no statistically significant difference between the wound areas of TA and Carbopol® containing 1% and 2% acemannan. Out of all of the treatments, Carbopol® containing 0.5% acemannan showed the greatest rate of wound healing. Carbopol® containing 0.5% acemannan significantly promoted oral wound closure when compared with TA, plain Carbopol®, and Carbopol® containing 1% and 2% acemannan ($P<0.05$) after 7 days of treatment (Fig. 3).

The histological study revealed that the wound edges of the acemannan-treated group were covered by epithelium (Fig. 4B); whereas in the normal saline-treated group, epithelium had only initially migrated into the wound edges (Fig. 4A). The tissues from the acemannan-treated group (Fig. 4D) also exhibited more rete ridges and dermal papillae than those in the normal saline-treated group (Fig. 4C). However, the thicknesses of the epithelium and keratin layers in all groups were not different (Figs. 4: A – D). In the connective tissue layers, the acemannan-treated group (Fig. 4F) showed relatively fewer inflammatory cells and more fibroblasts than the normal saline-treated group (Fig. 4E).

Discussion

Wound healing is a complex process. It is a response to injury aimed at reconstructing damaged tissue and requires precise coordination of connective tissue repair, re-epithelialization, and angiogenesis. To generate new tissue and heal the wound, fibroblasts not only proliferate to increase cell numbers, but also produce several extracellular matrix proteins and growth factors (1).

Aloe vera has long been used as a traditional medicine for burns and skin wounds (6). Acemannan, the major polysaccharide in *Aloe vera*, has been suggested to have a role in wound healing because of its immunostimulative effect through activation of macrophages in the

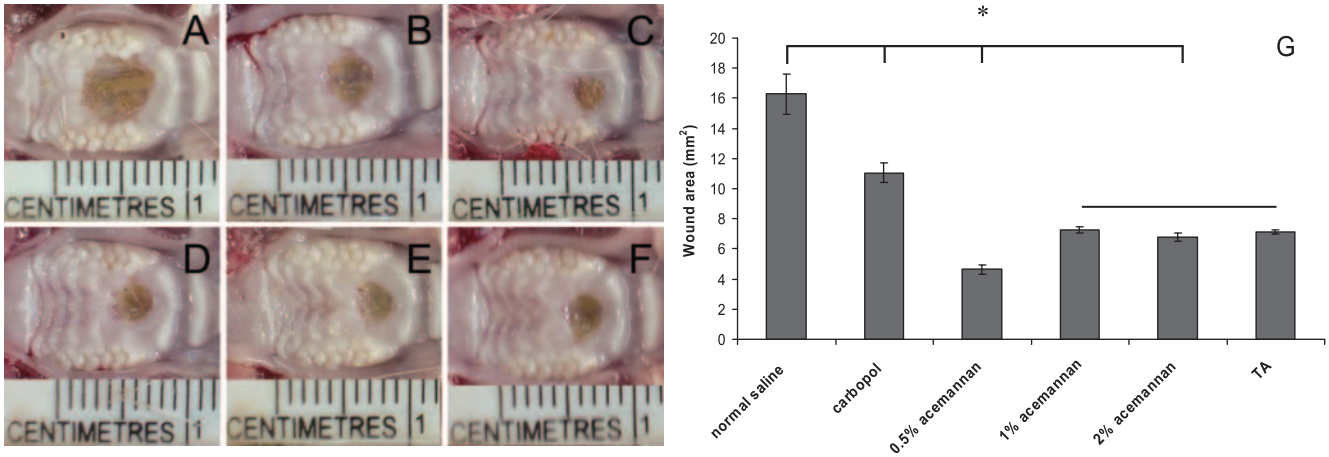

Fig. 3. Representative day 7 wounds on the hard palate of rats that received normal saline (A); plain Carbopol® (B); Carbopol® + 0.5% acemannan (C), Carbopol® + 1% acemannan (D), or Carbopol® + 2% acemannan (E); and triamcinolone acetonide (TA) (F). Wound areas on the hard palate of rats after receiving different agents for 7 days were evaluated by Image-Pro® Plus software. The Carbopol® + 0.5% acemannan–treated wound showed significantly smaller wound size as compared with normal saline, triamcinolone acetonide, and plain Carbopol® (G). *Significant difference between each group; $P<0.05$, n = 5.

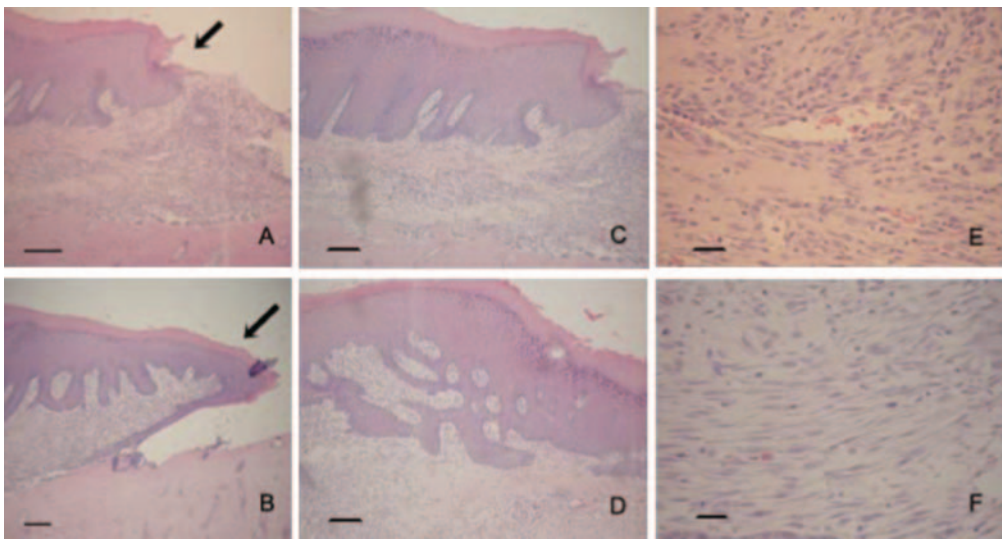

Fig. 4. Histopathological micrographs of wounds at day 7 of the experiment (H&E stain); normal saline–treated group (A, C, E), and Carbopol® + acemannan–treated group (B, D, F). Arrows indicate the re-epithelization front. Carbopol® + acemannan–treated wounds presented more fibroblast infiltration and rapid re-epithelization as compared with normal saline–treated wounds. bar = approximately 200 μm in A, B; 150 μm in C, D; and 50 μm in E, F.

inflammatory phase (10, 11, 17).

In this study, we investigated the potential of acemannan in other phases of wound healing, specifically the proliferative and extracellular matrix deposition phase. The proliferation and expression of some extracellular matrix proteins and growth factors were detected. [^{3}H]-Thymidine incorporation assay was used to evaluate cell proliferation. This assay is an accurate and simplified method to study proliferation by measuring the incorporation of radioactive precursors of DNA (18). Our data revealed that acemannan significantly induced GF proliferation. This finding is consistent with a previous study that *Aloe vera* gel crude extract has the ability to stimulate proliferation of diabetic skin fibroblasts (19). The acemannan at 16 mg/ml, the maximum concentration used in this study, was not toxic to GFs. This data corresponded with a previous study that reported no significant toxicity in oral administration of acemannan to rats for 14 days at 5% of the diet and to dogs for 90 days at up to 1,500 mg/kg per day (20).

Wound healing is mediated by several growth factors that are secreted in response to injury. Among the many growth factors secreted from fibroblasts, KGF-1 is strongly upregulated after an injury (21). Topical application of KGF-1 has been shown to accelerate the rate of re-epithelialization and increase the thickness of newly formed epidermis (22). Another important growth

factor is VEGF, which plays an important role in new blood vessel formation (23). Delay of full-thickness skin wound healing was found in VEGF-deficient mice (24). Topical application of VEGF has been reported to stimulate diabetic wound healing through increased angiogenesis (25).

The effects of acemannan on KGF-1 and VEGF secretion were examined by ELISA. The results showed that acemannan significantly stimulated the KGF-1 and VEGF synthesis from GFs. Previous studies have reported that acemannan stimulated macrophage and dendritic cell cytokine production (10, 26). Our data demonstrated that acemannan also enhanced production of growth factors from GFs.

Type I collagen is the main protein in gingival tissue. Collagen plays an important role in connective tissue healing by providing tissue strength and an extracellular matrix framework for cell adhesion and migration (2). Our data revealed that acemannan significantly enhanced type I collagen synthesis. In addition, effects of acemannan on growth factors and collagen expression still remained after 48 h of exposure.

From the in vitro data obtained in this study, punch biopsy wounds from the hard palate of rats were used to further evaluate the effect of acemannan on oral wound healing. Carbopol® 934P NF, a high molecular weight poly(acrylic acid), has been approved by the U.S. Food and Drug Administration (FDA) for use in oral and topical dosage forms (27). In addition, Carbopol® 934P NF has a mucoadhesive property (27). Although plain Carbopol® has not been used as a wound healing promotion agent, our data revealed that it significantly enhanced wound healing as compared with normal saline, the negative control. This effect resulted from its mucoadhesive property that acted as a wound dressing to prevent contamination and irritation (28).

Triamcinolone acetonide (TA) (0.1%) in dental paste is an anti-inflammatory steroid. It has been approved by the FDA for the relief of symptoms of any inflammatory condition in the mouth (29). Our data showed that Carbopol® containing 0.5% acemannan significantly reduced oral wound areas as compared with TA. Moreover, the animals that received Carbopol® containing 1% and 2% acemannan showed the same rate of wound healing as for TA. The optimum concentration of acemannan for oral wound healing should be 0.5% (w/w). The possible explanation for this effect could be that TA only plays a role in the inflammatory phase, whereas acemannan may be mainly involved in the proliferative and extracellular matrix synthesis phase of wound healing. This accelerated effect on oral wound healing is consistent with a previous study indicating that acemannan stimulated wound healing in the mouse footpad model (17).

Additionally, the histological data demonstrated that the acemannan-treated group had more rapid re-epithelization and more rete ridges and dermal papillae when compared with the negative control group. These connective tissue papillae provide vascular and nervous supply to the epithelium (30), which may be necessary for wound healing and maintaining the epithelium-connective tissue physiological structure. Histological data also revealed increased epithelialization in the acemannan-treated groups compared to that of the normal saline-treated group. This could be the paracrine effect of KGF-1 secreted by fibroblasts on epithelial growth (21). However, the direct effect of acemannan on epithelial proliferation and differentiation could not be excluded. Many plant polysaccharides such as *Phellinus gilvus* and *Opuntia ficus-indica* have been reported to have the capacity to enhance wound healing (4, 5). We conclude that acemannan accelerated the proliferative and extracellular matrix synthesis phase of oral wound healing via proliferation and upregulation of KGF-1, VEGF, and type I collagen expression in gingival fibroblasts.

At day 3 after treatment, Carbopol® containing 0.5%, 1%, and 2% acemannan and TA did not significantly reduce the wound area when compared with that in the normal saline-treated group. A possible explanation for this is that the first 3 days of wound healing is the inflammatory phase of wound healing. In this phase, only macrophages and white blood cells play major roles in clearing of bacteria and cell debris (1). While the second phase of wound healing, cell proliferation and extracellular matrix formation, would start around day 4 (1). This corresponded with our in vitro and in vivo data that suggest that acemannan stimulates fibroblast proliferation and type I collagen expression; and the reduction of wound area of experimental animals at day 7 after treatment.

Within the limitations of our data, the precise mechanism whereby acemannan induces proliferation and gene expression in gingival fibroblasts is still unknown. Based on the molecular weight and sugar structures of acemannan, we thought acemannan should bind to its receptor on the cell surface and induce the intracellular signaling pathway. Interestingly, the mannose receptor family — which is comprised of the mannose receptor, the M-type phospholipase A2 receptor DEC-205/gp200-MR6, and Endo180/uPARPAP — has been identified (31). These transmembrane receptors contain an N-terminal cysteine-rich domain, a fibronectin type II domain, multiple C-type lectin-like domains (CTLDs), and a C-terminal cytoplasmic domain (32). The CTLDs recognize polysaccharide chains terminating with

mannose, fucose, or *N*-acetylglucosamine. After binding to their ligands, the ligand–receptor complex would ingest and subsequently dissociate the ligands in the cell (31). To confirm our speculation that the mannose receptor could be the candidate acemannan receptor, future study is still required.

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