Effects of (1→3),(1→6)-β -D-Glucan on Wound Healing

Hyun Joo Son

Department of Medical Science
The Graduate School, Yonsei University

Effects of (1→3),(1→6)-β -D-Glucan on Wound Healing

Directed by Professor Jong Chul Park

The Master's Thesis
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the
degree of Master of Medical Science

Hyun Joo Son

December 2005

This certifies that the Master's Thesis of Hyun Joo Son is approved.

_____

Thesis Supervisor: Jong-Chul Park

_____

Dong Kyun Rah

_____

Myoung Hee Kim

The Graduate School
Yonsei University

December 2005

ACKNOWLEDGEMENTS

I've just finished the arduous, but fruitful time and now get a result here. There are so many people that I appreciate for supporting me. And without them, I couldn't have been here.

First of all, I want to thank my adviser, prof. Jong-Chul Park, who is delicate and had given me many chances of diverse experiences. And I also really thank my thesis directors, prof. Dong Kyun Rah and prof. Myoung Hee Kim for unstinted advices. I would like to add my own appreciation to all professors of department of Medical Engineering. There are also two professors that I want to thank, prof. In-Seop Lee and Kwon-Yong Lee. And I appreciate sincere help of Doc. Dong Hee Lee. Thank you very much.

I want to return special thanks to my laboratory colleagues, Hyun Sook, Hye Ryeon, Mi Hee, Yeon I, Jin Hyun, Hyun Jeong, Min Sub and Bong Joo and Dong Wook in Japan. Without them, there couldn't have been this thesis. Thank you and love you all. And I owe all members of department of Medical Engineering, Metal Engineering and Sejong University so much.

I have many best friends to appreciate, Mok Yeon, Paul, Sang kyun, Tae Yun, Sung Jin, Hyung Seop, Young Ran, Ddol Ddol, Lian, good sisters, Seung A, Ja Young and Dari. And finally I really thank my parents and sister Hyun Kyung and Hye Jeong. I love you.

sincerely yours

Hyun Joo Son

Contents

Figure legends

Table legends

Abbreviations

ECM : Extracellular matrix

TNF-α : Tumor necrosis factor-α

IL-6 : Interleukin-6

TGF-β : Transforming growth factor-β

DMEM : Dulbecco's modified Eagle's medium

FBS : Fetal bovine serum

CCD : Charge-coupled device

ELISA : enzyme-linked immunosorbant assay

SD : Sprague-Dawley

BGC : Beta-glucan collagen

PBS : Phosphate buffered saline

H&E : Hematoxylin and eosin

Abstract

Effects of (1→3),(1→6)-β-D-Glucan on Wound Healing

Hyun Joo Son

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Jong-Chul Park)

β-Glucan is a natural product of glucose polymer, which has immune stimulatory activity, especially effective on wound healing. Relating with wound healing activity of β-glucan, two major mechanisms can be hypothesized as direct and indirect pathways. First hypothesis is that β-glucan directly signals fibroblasts to migrate and proliferate, through their receptors on fibroblasts. It was already reported that there are at least two β-glucan binding sites on normal human dermal fibroblasts other than immunocytes such as mammalian macrophage. Second hypothesis is that β-glucan, treated after the occurrence of wound, activates macrophages to release some cytokines and growth factors, which signal fibroblasts to migrate into wound sites and proliferate. Among those cytokines and growth factors secreted from macrophages, pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) or transforming growth factor-β (TGF-β) are especially related with fibroblast activities in wound healing. In the present study, we evaluated the direct and

indirect effects of (1→3),(1→6)-β-D-glucan on fibroblast for wound healing process through *in vitro* and also *in vivo* tests.

The results of fibroblast proliferation and migration assay showed that (1→3),(1→6)-β-D-glucan have effect on the migration of adult human dermal fibroblasts, but no positive effect on proliferation. And β-glucan activated macrophages to upregulate the TNF-α and TGF-β1 secretion, but no significant difference was shown in the amount of IL-6. TNF-α is known to be mitogenic for fibroblasts and stimulate collagen biosynthesis. And TGF-β1 stimulates fibroblasts to proliferate, migrate and promote production of ECM during wound healing. These results suggested that β-glucan have effect on wound healing through cytokines and growth factor production by macrophages. In the result of i*n vivo* wound healing assay, β-glucan treated wounds showed the faster contraction than saline or collagen treated wounds. This result means that topical administration of (1→3),(1→6)-β-D-glucan was useful to promote wound healing.

In conclusion, these results suggest that (1→3),(1→6)-β-D-glucan could be helpful to wound healing by fibroblast activation through both direct and indirect pathways.

Key Words : (1→3),(1→6)-β-D-glucan, wound healing, fibroblast, proliferation, migration, macrophage, cytokine, growth factor

Effects of (1→3),(1→6)-β-D-Glucan on Wound Healing

Hyun Joo Son

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Jong-Chul Park)

1. Introduction

1.1 Wound healing processes

Wound healing is a highly complex physiological process that involves the interaction of various cells with diverse phenotypes such as macrophages, fibroblasts, and it is mainly mediated by immune reaction.[1-4] Following tissue injury, blood vessels are disrupted resulting from extravasation of blood components. This is followed by platelet aggregation and blood coagulation resulting in the generation of a fibrin-rich clot that fills the discontinuity in the injured tissue and reestablishes homeostasis.[5,6] Subsequently, the migration of inflammatory cells to the wound site begins and these cells debride the necrosed tissue of wound. This marks the beginning of the inflammatory phase of wound healing. During the initial inflammatory phase, fibroblasts migrate to wound sites where they proliferate, synthesize and later remodel new extracellular matrix (ECM) materials, of which collagen is the main component.[5,7] This is followed by the formation of new blood vessels, or angiogenesis, within the newly forming granulation

tissue. Granulation tissue formation includes the migration, proliferation and differentiation of dermal fibroblasts, with degradation and resynthesis of the extracellular matrices.[7] This is then transformed to the final product of the healing process, a scar. A scar is a relatively avascular and a cellular mass of collagen which serves to restore tissue continuity, strength and function.[8] Because the residual scar is not identical to the surrounding dermis, wound healing should therefore be defined as a pathophysiological process. Since the migration of fibroblasts is thought to be an essential event of the initial healing process and also, it has intimate relation to scar formation, the effects of various wound healing promoting factors on the migration fibroblasts have been investigated using chemotaxis assays or wound models.[9,10]

1.2 Wound healing mechanisms

Fibroblasts play a central role in wound healing by producing the ECM molecules that ultimately replace the damaged tissue, by first migrating into the wound sites and then proliferating followed by depositing new matrix and restructuring the tissues.[8] As part of the process of closure, wounds may undergo contraction, giving rise to scaring and loss of function.[11-13]

In the wound healing mechanisms, there have been two major hypotheses concerned about fibroblast activities including migration, proliferation and collagen formation. One is that after the occurrence of wound, activated macrophages release cytokines and growth factors, which signal fibroblasts to migrate into wound

sites where they proliferate and constitute new ECMs.[14] It is generally accepted that wound healing is mediated by immune reaction and macrophage is a pivotal cell in wound repair.[15] In actuality it has been reported that after the occurrence of wound, macrophages are activated and secrete a plethora of biologically active substances, such as nitric oxide, proinflammatory cytokines including tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6), or some growth factors such as Transforming growth factor (TGF-β).

Another hypothesis about fibroblast activities in wound healing is some chemicals from wound might directly signal fibroblasts to migrate, proliferate followed by collagen formation through their specific receptors on the surfaces of fibroblasts.

1.3 Cytokines and growth factors released from macrophages

Macrophage has a pivotal role in modulating the repair process, mediating phagocytosis, and producing a myriad of cytokines and growth factors to control wound healing and cell recruitment as well as proliferation.[15-17] Cytokines and growth factors are involved in both the inflammatory and proliferate phases of wound healing processes. Pro-inflammatory cytokines including TNF-α, IL-1β, IL-2 and IL-6 are widely expressed at the early phases of wound healing, and growth factors such as TGF-β are also secreted extensively. Among these cytokines and growth factors released from macrophages, some cytokines and growth factors are known to modulate the fibroblasts activities such as migration,

proliferation and ECM formation (Table 1).

1.3.1 Interluekin-6

IL-6 is a frequently encountered proinflammatory cytokines, involved in a variety of immunological functions as well as interaction with a variety of target cells. IL-6 has been shown to promote terminal differentiation of proliferating B-cells to plasma cells, stimulation of antibody secretion, and inducing synthesis of acute-phase proteins. IL-6 is secreted primarily by monocytes and macrophages and always found in increased levels at sites of inflammation.

1.3.2 Tumor necrosis factor-α

The name of TNF-α was derived from the cytostatic or cytocidal characteristics for tumor cells *in vitro*. However, normal cells as well as tumor cells have TNF receptors. TNF-α, mainly produced by macrophages, is a major proinflammatory cytokine which is present at elevated levels early after wound and is believed to play important roles in the process of repair. It is mitogenic for fibroblasts and, in addition, stimulates collagen and collagenase biosynthesis and prostaglandin E_2 release.[18-20] It can also stimulate angiogenesis and induce the secretion of factors that modulate a variety of other cellular functions as well.[21]

Table 1. Cytokines and growth factors released from macrophages in relation to fibroblast activities during wound healing processes.

Growth factors /cytokines	Abbreviation	Source	Functions
Interleukins	IL-1, etc	Macrophages, lymphocytes, many other tissues and cells	Differentiation of proliferating B-cells to plasma cells, Fibroblast proliferation, neutrophil chemotaxis
Tumor necrosis factor	TNF	Macrophage, mast cells, T lymphocytes	Fibroblast proliferation, collagen biosynthesis, angiogenesis
Transforming growth factor β	TGF-β	Platelets, neutrophils, lymphocytes, macrophages, many other tissues and cells	Fibroblast proliferation, chemotaxis, collagen metabolism, angiogenesis

1.3.3 Transforming growth factor-β

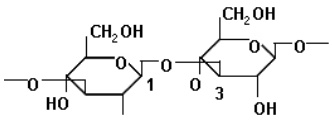
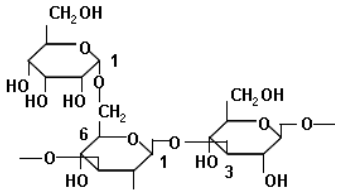
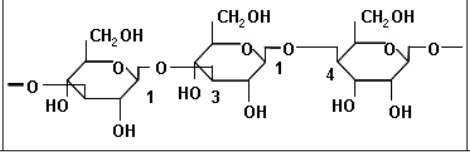
TGF-β was originally isolated as a stimulant of anchorage-independent cellular proliferation in soft agar.[22] Several subtypes have subsequently been identified including TGF-β1 and TGF-β2 with varying affinity for the several TGF-β receptors. There are no major differences in terms of function among the various subtypes.[23,24] TGF-β has been isolated from a number of cells and tissues including platelets, macrophages, lymphocytes, bone, and kidney and appears to be nearly ubiquitously produced.[25-29] Essentially all cells have receptors for TGF-β and can at least theoretically respond to it. TGF-β is chemotactic for macrophages and stimulates fibroblasts to proliferate, migrate and promote production of ECM during the critical events of wound healing.[30,31] TGF-β may be the most potent stimulant of collagen synthesis and it, in addition, decreases the stimulatory effect of other factors on collagenase activity.[32-35] TGF-β also upregulates fibronectin and proteoglycan synthesis by fibroblasts and fibronectin synthesis by keratinocytes.[36,37] It may participate in wound contraction as we11 and also has the ability to organize matrix and be involved in scar remodeling.[38]

1.4 β-D-Glucans

β-D-glucans are polymers of glucose that mainly construct the outer cell wall of fungi and certain bacteria.[39-41] Beta-glucans have three structures according to the types of chain linkage, which are linear (1→3) or (1→3),(1→4)-β-D-glucans and (1→6) branched (1→3)-β-D-glucans (Table 2). And according to the structures, properties and main resources are little different from each others.

β-D-glucans are well known as a wound healing accelerator. Glucans have been demonstrated to have immune stimulatory activity, especially to enhance wound healing by increasing macrophage infiltration into the injury sites, stimulating tissue granulation, collagen deposition, and reepithelialization.[42-45] It has been reported that topical or systemic glucan administration enhances wound healing.[42,43,46-49] Delatte et al. have reported on the effectiveness of a β-glucan collagen preparation in the treatment of partial thickness burn in pediatric patients.[45] They reported that pediatric burns can be effectively treated with β-glucan-collagen mixtures and that this preparation markedly simplified wound care and significantly decreased post-injury pain.[45] These observations suggest that β-glucans and related immunomodulators may be useful adjuncts for healing, particularly in burn wounds. Furthermore Anders et al. demonstrated that (1→3)-β-D-glucan

Table 2. Classification of β-D-Glucans

Resource	Structure	Description
Bacteria		Linear (1→3)-β-D-glucan (Curdlan)
Yeast		Short (1→6) branched (1→3)-β-D-glucan (Schizophyllan)
Fungi		Long (1→6) branched (1→3)-β-D-glucan (Betafectin™)
Cereal		Linear (1→3),(1→4)-β-D-glucan (from oats, barley, rye)

induced the release of a number of inflammatory cytokines such as TNF-α, IL-1β and IL-6 from macrophages.[50] However, until now these kinds of investigations cannot have clarified the pathways of the effects of β-D-glucans on wound healing *in vitro,* and also *in vivo* systematically. Up to the present, some clinical application cases have been reported, but there are no verified explanations about how β-glucan affects on wound healing.

Relating with hypotheses of wound healing mechanisms for fibroblast activities, two ways of evaluation effects of β-D-glucans on wound healing can be considered, which is indirect through various cytokines released from macrophages and/or a direct influence of β-D-glucans on fibroblasts. Kougias et al. have reported the presence of at least two glucan binding sites on normal human dermal fibroblasts other than immunocytes like mammalian macrophages.[25] Putting various reports together, both pathways have possibilities to explain the mechanisms for wound healing.

1.5 Objectives of this study

The purpose of this investigation is the evaluation of effects of (1→3),(1→6)-β-D-glucan on wound healing both *in vivo* and *in vitro*. (1→3),(1→6)-β-D-glucan is composed as (1→6) branches which are connected to linear (1→3)-β-D-glucan polymer and mainly extracted from the outer cell walls of the fungus and yeast, such as *Aureobasidium*, the black yeast. (1→3),(1→6)-β-D-glucan is colorless and odorless and has relatively low viscosity. It is also be divided into soluble and insoluble groups in water and well known to have the medical activity.[51]

In this study, on the basis of two hypotheses about fibroblast activities in wound healing, the direct and indirect effects of (1→3),(1→6)-β-D-glucan on fibroblasts were tested. We conducted the cell proliferation and migration assay *in vitro* and next, quantitative analysis of the cytokines and growth factor from macrophages activated by (1→3),(1→6)-β-D-glucan. Finally, we executed *in vivo* test by direct applications to wounds of rats.

2. Materials and methods

2.1 (1→3),(1→6)-β-D-glucan

Water soluble (1→6) branched (1→3)-β-D-glucan was extracted from *Aureobasidium pollulans*, a kind of black yeast. β-glucan was obtained as a powder form among which about 95 % is β-glucan. The powder β-glucan was stocked as 10 mg/ml dissolved in distilled water or saline followed by filtering with syringe. Stocked β-glucan was colorless and odorless with low viscosity.

2.2 Cells and cell cultures

Adult human dermal fibroblasts (aHDFs) were isolated from adult human dermis by collagenase digestion and from five to ten passages were used. The mouse macrophage-like cell, RAW 264.7 was obtained from American Type Culture Collection (Rockville, MD, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma Co., St. Louis, MO, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS, Sigma Co., St. Louis, MO, USA) and 1% antibiotic antimycotic solution (Sigma Co., St. Louis, MO, USA) at 37°C in a humidified 5% CO_2 incubator.

2.3 Cell proliferation assay

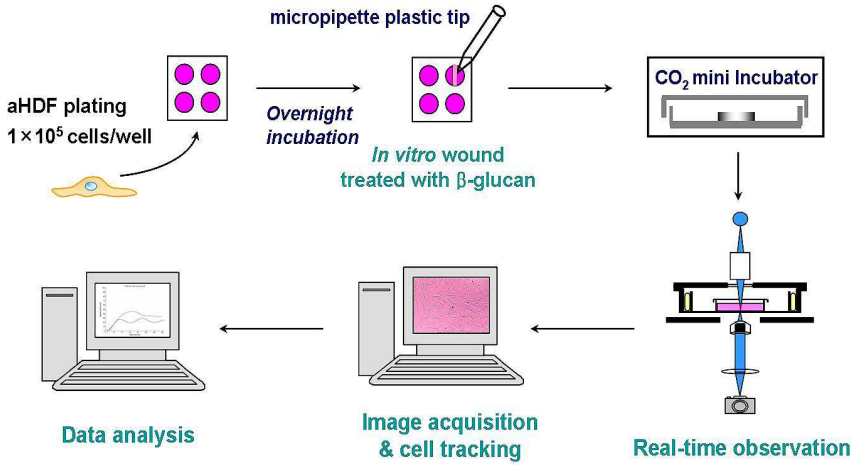
Culture wells (24-well culture plate, Falcon, NJ, USA) were inoculated with 1 ml growth medium containing 2×10^4 cells of aHDFs, co-treated with (1→3),(1→6)-β-D-glucan as the concentrations of 0.1, 0.5, 1 mg/ml, estimated not to have cytotoxicity, and incubated for 3 days in CO_2 incubator. The proliferated cells were quantified using MTT assay which measures the mitochondrial dehydrogenase activity of living cells, based on the reduction of the yellow tetrazolium salt-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Co., St. Louis, MO, USA) by metabolically active cells to form insoluble purple formazan crystals. After cell culture for 3 days, 100 μm of MTT reagent was added to the samples. After 4 hr of incubation at 37°C in humidified atmospheres of 5 % CO_2, the produced formazan crystals were dissolved in 400 μl of dimethyl sulfoxide and the solution was transferred to a 96 well plate (Falcon, NJ, USA). The absorbance of the resulting solution was measured using an ELISA reader (Spectra Max 340, Molecular Device Co., Sunnyvale, CA, USA)at a wavelength of 570 nm. The absorbance is directly proportional to the mitochondrial activity which is related to the number of living cells present in the culture wells.

2.4 *in vitro* wound healing and cell migration assay

aHDFs (1 x 10^5 cells/ml) were seeded in the 4-well chambered cover-glass slide (NUNC, Roskilde, Denmark) and grown to confluence overnight. Monolayers were wounded using a plastic micropipette tip and 1 mg/ml β-glucan was treated to the attached cells.[52] The cells were incubated in the self-designed CO_2 mini-incubator placed on the microscope stage for 36 hours, and visualized for migration of cells into denuded space by the charge-coupled device (CCD) camera (Electric Biomedical Co. Ltd., Osaka, Japan) attached to the inverted microscope (Olympus Optical Co. Ltd., Tokyo, Japan) (Figure 1).

2.4.1 CO_2 mini-incubator

In order to incubate the cells on the stage of an inverted microscope, a CO_2 mini-incubator with 150 mm in length, 130 mm in width and 40 mm in height was self-made, and a double-layered acrylic plate with 5 mm in thickness was covered on it. Two temperature sensors and heating-tape were used to monitor and maintain the temperature of the mini-incubator to 37°C. The mini-incubator was connected with a CO_2 incubator and a mini-pump to supply CO_2, and placed on the stage of an inverted microscope. The inverted microscope connected with a color CCD camera conveyed the images from the mini-incubator as a real time-mode and a frame grabber card in computer captured these images at regular intervals and memorized them as bmp image files.



F igure 1. Procedure for *in vitro* wound healing and cell migration assay

2.4.2 Image processing for single cell tracking

The captured 8-bit color images was converted into gray scale images after incorporating into the image analysis software programmed by MATLAB V5.3 (MathWork Inc., USA) and Visual Basic V6.0 language (Microsoft, USA). The edge point of each cell was detected and then the center of the detected edges was determined. The migration of the center was recorded and the time lapse and average graphs of cell movement speed was obtained. Unlike the preexisted systems, this cell tracking system has an advantage on the time lapse tracking of single cells.

2.5 Cytokine and growth factor secretion assay

1×10^5 cells of RAW 264.7 were seeded in 48 well culture plate (Falcon, NJ, USA) and incubated with or without (1→3),(1→6)-β-D-glucan (0.5 and 1.0 mg/ml) for 24 hr in CO_2 incubator. The quantitative levels of TNF-α, IL-6 and TGF-β1 in cell supernatant were determined by enzyme-linked immunosorbant assay (ELISA) kits following the manufacturer's instructions and the results were expressed as mean amount per 10^3 macrophages.

2.6 *In vivo* wound healing assay

2.6.1 Animals

Male Sprague-Dawley (SD) rats weighing over 250 g were used for this study.

2.6.2 Preparation of collagen and β-glucan solutions

Collagen (Sigma Co., St. Louis, MO, USA) was resolved in 70% ethanol and completely frozen, followed by freeze-drying. This procedure was repeated again and then, the collagen was resolved in 0.2% acetic acid (Sigma Co., St. Louis, MO, USA). (1→3),(1→6)-β-D-glucan was resolved in saline and the solution was filtered using syringe filter. Beta-glucan collagen (BGC) mixture was prepared as final concentrations as 5 mg/ml respectively.

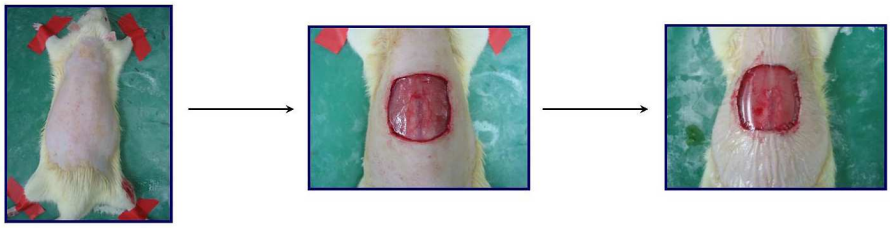
2.6.3 Wound contraction, animal grouping and drug administration

The rats were anaesthetized with Ketara (Yuhan Co., Seoul, Korea) and their back was shaved. The hair of back was removed clearly by using Niclean cream (Ildong Co., Seoul, Korea). 2.5 cm × 2.5 cm full thickness of wounds were made on the backs of rats. The wounds of negative control were applied with 0.8 ml of saline and those of positive control were applied with same volume of 5 mg/ml collagen solution. For experimental rats, 0.8 ml of BGC

mixture was treated and then, transparent film, Tegaderm (3M Co., St. Paul, MN, USA) was applied over the wounds (Figure 2). The image of each wound was daily obtained and the area of wound was measured by computer image analyzing software, Scion Image.

2.7 Statistical analysis

All results were expressed as a mean±standard deviation and analyzed by Student *t*-test (Excel 2003, Microsoft, WA USA). Statistical significance was considered at $p < 0.05$.



Removing hair Full-thickness wound Reagent treatment

Figure 2. Procedure for *in vivo* wound healing assay

3. Results

3.1 Effect of (1→3),(1→6)-β-D-glucan on fibroblast proliferation

The result of cell proliferation assay showed that there was no significant difference in proliferation rate between (1→3),(1→6)-β-D-glucan-treated and non-treated aHDFs (Figure 3).

3.2 Effect of (1→3),(1→6)-β-D-glucan on fibroblast migration

3.2.1 Morphological observation of migrating aHDFs

The images of migrating aHDFs on the denuded areas, which were at an interval of 12 hr were shown in Figure 4. The arrows point the cells in edges of denuded areas. In images at 12 hr, aHDF cells of 1 mg/ml β-glucan treated group were migrating faster than those of non-treated group and in images at 24 hr, already many cells in β-glucan treated group had been migrated up to the center of denuded area. Finally after 36 hr, almost all of the denuded area of β-glucan treated group was covered with cells which migrated from both sides of denuded areas or proliferated after migration.

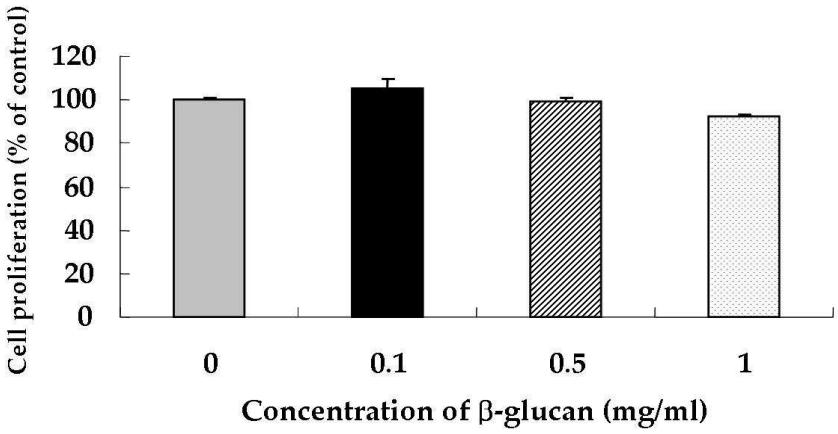


Figure 3. Effect of (1→3),(1→6)-β-D-glucan on proliferation of aHDF cells. The results are shown as a mean ± standard deviation (n = 3). The data is analyzed by Student t-tests, and the values are significantly ($p< 0.05$) different from the non-treated control. There was no significant difference between β-glucan-treated and non-treated groups.

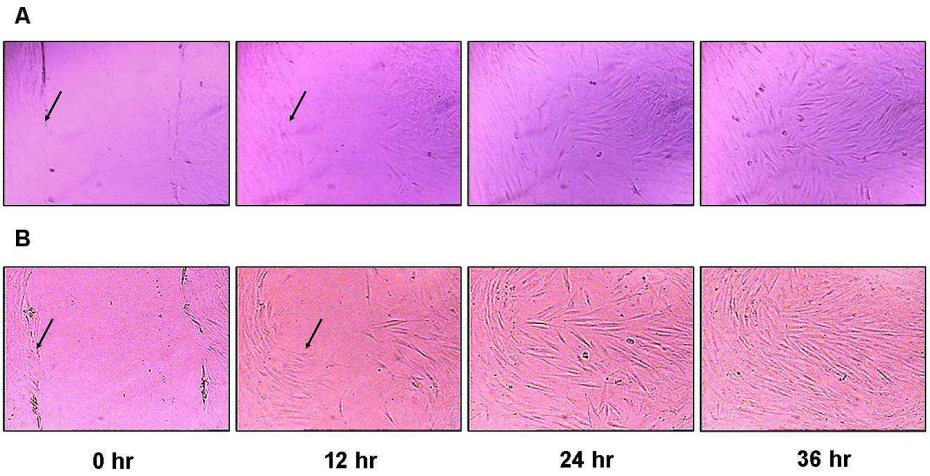


Figure 4. Morphological observation of migrating aHDFs at an interval of 12 hr. (A) is non-treated control group and (B) is 1 mg/ml (1→3),(1→6)-β-D-glucan treated experimental group observed at 40× magnification. The denuded area of β-glucan treated group was covered with aHDFs faster than that of control.

3.2.2 Migration speed of aHDFs

The real time migration speed of β-glucan treated and non-treated aHDFs was shown as Figure 5. A dotted line means non-treated control group and a solid line points 1 mg/ml (1→3),(1→6)-β-D-glucan treated experimental group. The speed of non-treated group increased for 9 hr from start and till 36 hr, changed from 20 to 30 ㎛/hr, while that of 1 mg/ml β-glucan treated group gradually increased up to approximately 17 hr and remained faster than that of control. Furthermore, the average migration speed of the 1 mg/ml (1→3),(1→6)-β-D-glucan treated cells was 40.95 ㎛/hr, while that of the non-treated group was 30.95 ㎛/hr (Figure 6).

3.3 Effect of (1→3),(1→6)-β-D-glucan on macrophage activation

3.3.1 Cytokine secretion

The amount of TNF-α secreted from 10^3 RAW 264.7 cells treated with (1→3),(1→6)-β-D-glucan increased compared to that of the non-treated control (Figure 7). Especially 1 mg/ml β-glucan treated group (4.28 pg/ml/10^3 cells) showed the increase of over three fold to control group (1.25 pg/ml/10^3 cells). But there was no significant difference in the amount of IL-6 between β-glucan treated (1.47 and 2.26 pg/ml/10^3 cells) and non-treated groups (2.49 pg/ml/10^3 cells) (Figure 8).

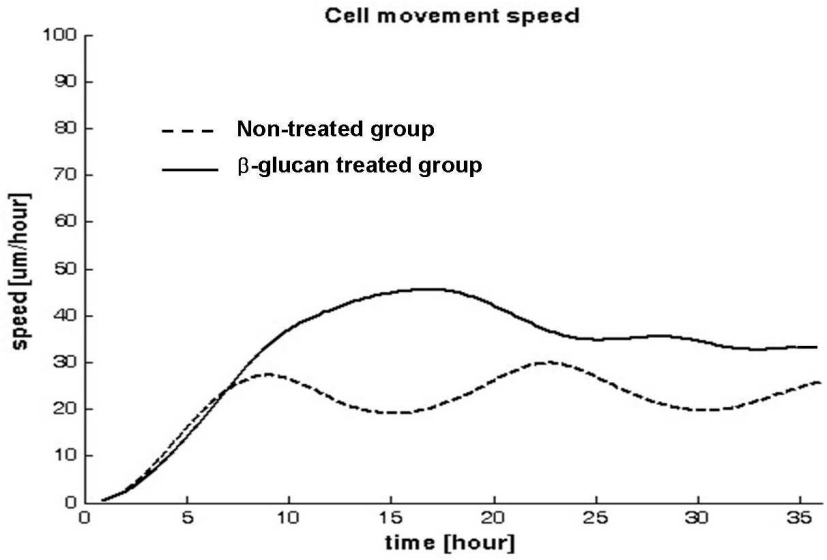


Figure 5. Real time migration speed of β-glucan treated and non-treated aHDFs. A dotted line for non-treated control group and a solid line for 1 mg/ml (1→3),(1→6)-β-D-glucan treated experimental group. β-glucan treated cells were migrated faster than control.

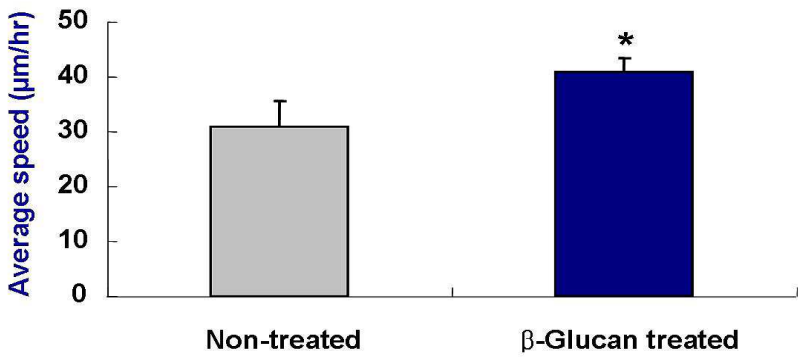


Figure 6. Average migration speed of β-glucan treated and non-treated aHDFs. Data are expressed as mean ± standard deviation (n=3). Analyzed by t-tests and statistical significance was considered as $p< 0.05$. The average migration speed of β-glucan-treated cells was faster than that of non-treated group.

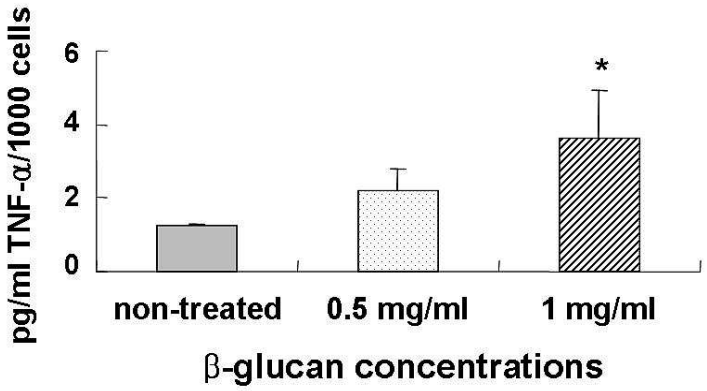


Figure 7. Amount of TNF-α released from 1,000 macrophage cells activated by 0.5, 1 mg/ml (1→3),(1→6)-β-D-glucan. Data are expressed as mean ± standard deviation (n=3). Analysis was performed by t-tests and statistical significance was considered as $p< 0.05$. The amount of TNF-α secreted from 10^3 RAW 264.7 treated with β-glucan, especially 1 mg/ml, was significantly increased compared to that of the non-treated control.

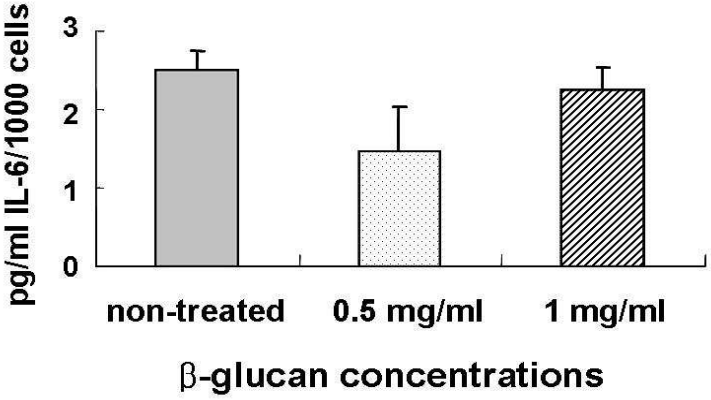


Figure 8. Amount of IL-6 released from 1,000 macrophage cells activated by 0.5, 1 mg/ml (1→3),(1→6)-β-D-glucan. Data are expressed as mean ± standard deviation (n=3). There was no significant difference in the amount of IL-6 between β-glucan treated and non-treated groups.

3.3.2 Growth factor secretion

The amount of TGF-β1 secreted from 10^3 RAW 264.7 cells treated with (1→3),(1→6)-β-D-glucan was significantly increased compared to that of the non-treated control as showed in Figure 9 ($p<0.05$). 0.5 mg/ml β-glucan treated group (0.095 pg/ml/10^3 cells) showed the increase of over nine fold to control group (0.010 pg/ml/10^3 cells) and 1 mg/ml β-glucan treated group (0.072 pg/ml/10^3 cells) also showed the increase of about seven fold.

3.4 *in vivo* effect of (1→3),(1→6)-β-D-glucan on wound healing

3.4.1 Photographical observation of wound healing

The photographical representation of contraction rate on different days of wounds treated with saline, collagen and BGC groups were shown in Figure 10. The wounds of BGC mixture treated rats were contracted faster than those of saline or collagen treated rats especially after day 6.

3.4.2 Rate of contraction

The contraction rates of wounds on different days were expressed as percentage of initial wounds (Figure 11). The contraction rate of BGC mixture treated wounds on day 3 and 14 (23.33 and 89.02%) was significantly higher than that of saline

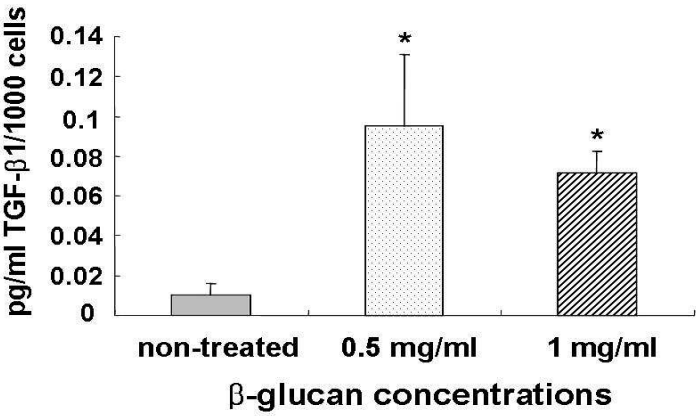


Figure 9. Amount of TGF-β1 released from 1,000 macrophage cells activated by 0.5, 1 mg/ml (1→3),(1→6)-β-D-glucan. Data are expressed as mean ± standard deviation (n=3). Analysis was performed by t-tests and statistical significance was considered as $p< 0.05$. The amount of TGF-β1 secreted from 10^3 RAW 264.7 treated with β-glucan was significantly increased compared to that of the non-treated control.

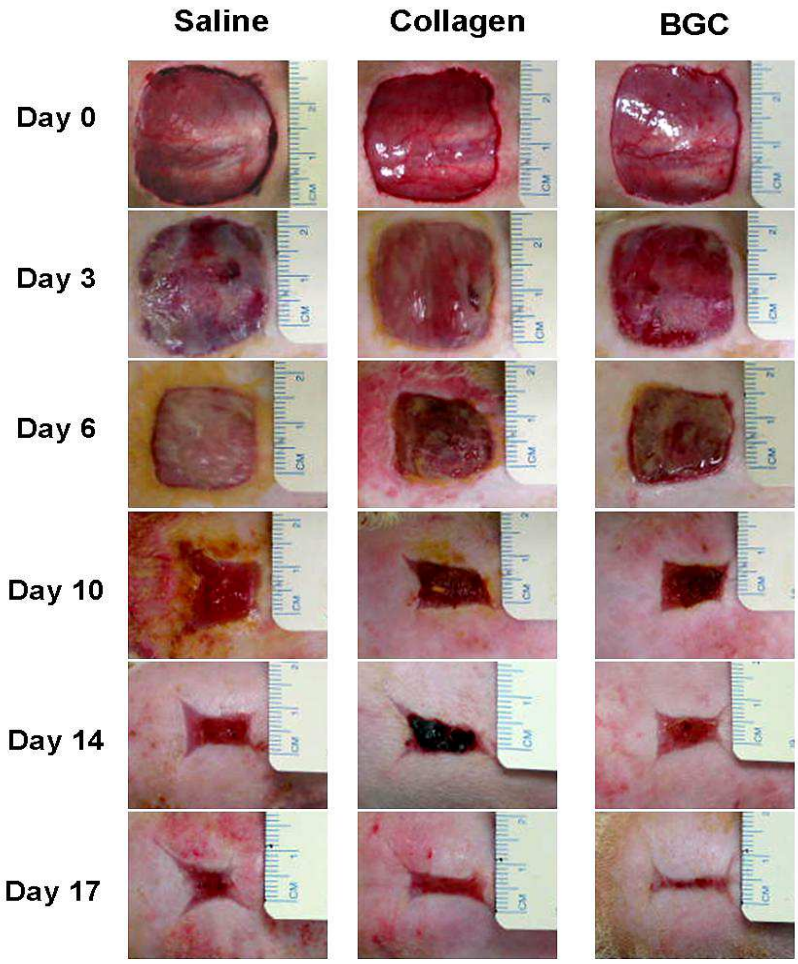


Figure 10. Photographical representation of contraction. Wounds were treated with saline, collagen and BGC mixture as written in the materials and methods, then photographs were taken at each days shown on the left. BGC mixture treated wounds contracted faster than saline or collagen treated wounds.

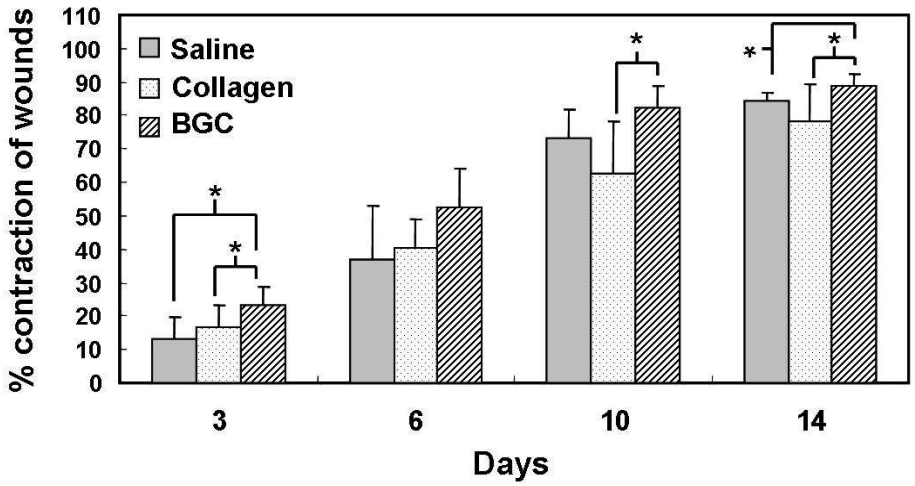


Figure 11. The rate of contraction in controls and experimental rats at different days. Values are expressed as mean ± standard deviation (n=6). The contraction rate of BGC mixture treated wounds on day 3, 10 and 14 was significantly higher than that of saline or collagen treated wounds.

(12.94 and 84.20%) or collagen (16.57 and 78.44%) treated wounds ($p<0.05$). Also, BGC mixture treated wounds (82.24%) were contracted significantly faster than collagen treated wounds (62.79%) on day 10 ($p< 0.05$). After day 14, the contraction rates of three groups was similar (data not shown).

4. Discussion

In order to evaluate the effects of (1→3), (1→6)-β-D-glucan on wound healing, the direct and indirect effects of (1→3), (1→6)-β-D-glucan on fibroblasts were tested on the basis of two hypotheses about fibroblast activities in wound healing. The cell proliferation and migration assay were conducted using aHDFs and the quantities of cytokines and growth factor secreted from macrophages, treated with (1→3), (1→6)-β-D-glucan was evaluated. Finally, we executed *in vivo* test by direct applications of (1→3), (1→6)-β-D-glucan to wounds of rats.

The cell proliferation assay showed that there was no increasing effect of (1→3), (1→6)-β-D-glucan on the proliferation of aHDFs. This result is consistent with the previous report that chitosan, which is a polysaccharide and known as a immunostimulator like glucan, showed almost no proliferate effect on fibroblast *in vitro*[54]. On the other hand, in the cell migration assay, the morphologies of aHDFs migration to denuded area showed that the area of (1→3), (1→6)-β-D-glucan treated group covered faster with cells than that of non-treated control. Since β-glucan did not have any effect on fibroblast proliferation was already mentioned, that result means β-glucan has an effect on fibroblast cell migration only. Also the cell tracking system used here could show the real time motility of fibroblast, therefore we checked the result of cell migration assay was not influenced by cell proliferation. And the average migration speed of the 1 mg/ml β-glucan-treated cells was also faster than that of non-treated group. From these results, we can speculate that fibroblasts have

receptors for β-glucan on their surface and through them β-glucan enhances the fibroblast migration, but not the fibroblast proliferation.

In the case of cytokine secretion, it was demonstrated that (1→3), (1→6)-β-D-glucan was able to stimulate the production of TNF-α in macrophages. The amount of TNF-α secreted from 10^3 RAW 264.7 treated with β-glucan, especially 1 mg/ml, was increased compared to that of the non-treated control. But there was no significant difference in the amount of IL-6 between β-glucan treated and non-treated groups. In the growth factor secretion assay, the amount of TGF-β1 secreted from 10^3 RAW 264.7 treated with β-glucan, both at the concentrations of 0.5 and 1 mg/ml, was also increased compared to that of the non-treated control. These results suggest that β-glucan influences on macrophage activation, particularly on TNF-α and TGF-β1 secretion from macrophages. TNF-α is known to be mitogenic for fibroblasts and stimulate collagen biosynthesis.[18-20] And TGF-β1 stimulates fibroblasts to proliferate, migrate and promote the production of ECM during the critical events of wound healing.[30,31] Thus, it could be suggested that β-glucan activates macrophages to release more TNF-α and TGF-β1, which then enhance the fibroblast activities in wound healing indirectly. However, stimulation with β-glucan did not induce the secretion of IL-6 and the production of this cytokine might be limited to certain maturation stages.

In vivo wound healing assay evaluated that BGC mixture treated wounds showed the faster contraction than saline or collagen treated wounds. Especially the initial contraction of BGC

mixture treated wounds was fast, which is important in wound healing in order to prevent the infection. Collagen is well known as an extracellular matrix and has been used as collagen matrix substitute dermis or artificial dermis, which is available for treating deep wounds.[55] Thus, the result that BGC mixture treated wounds contracted faster than collagen only treated wounds means that BGC mixture can be more effective on wound healing than collagen only. And this suggests that topical administration of (1→3), (1→6)-β-D-glucan was useful to promote wound healing.

In the present study, it was be suggested that (1→3), (1→6)-β-D-glucan can influence on wound healing by enhancing the fibroblast migration via a direct interaction with fibroblasts through the β-glucan receptors as well as activating macrophages to secret more TNF-α and TGF-β1 which subsequently induce of fibroblast activities. And this means that (1→3), (1→6)-β-D-glucan could be a candidate reagent for wound healing.

5. Conclusion

In this study, the direct and indirect effects of (1→3),(1→6)-β-D-glucan on fibroblasts activities in wound healing were evaluated.

Fibroblast proliferation and migration assay showed that (1→3),(1→6)-β-D-glucan have effect on the migration not the proliferation of adult human dermal fibroblasts. And β-glucan activated macrophages to upregulate the TNF-α and TGF-β1 secretion, but no significant difference was shown in the amount of IL-6 secretion. *In vivo* wound healing assay revealed that β-glucan treated wounds showed the faster contraction than saline or collagen treated wounds. These results suggest that (1→3),(1→6)-β-D-glucan affects on fibroblast activities diectly and indirectly via macrophage activation. Also (1→3),(1→6)-β-D-glucan is effective on wound contraction.

In conclusion, (1→3),(1→6)-β-D-glucan can be a candidate reagent for wound healing.

References

1. Bennett NT, Schultz GS. Growth factors and wound healing: biochemical properties of growth factors and their receptors. Am J Surg 1993;165:728-737.
2. Robinson CJ. Growth factors: therapeutic advances in wound healing. Ann Med 1993;25:535-538.
3. Hofer HP, Egger G, Bratschitsch G, Kutovetz EM, Petek W, Schaur RJ. Phagocyte activation and its implication in wound healing following trauma surgery. Med Sci Res 1994;22:533-534.
4. Steenfos HH. Growth factors and wound healing. Scand J Plast Reconstr Surg Hand Surg 1994;28:95-105.
5. Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med 1999;341:738-746.
6. Buffoni F, Banchelli G, Cambi S, Lgnesti G, Pirisino R, Raimodi L, et al. Skin wound healing: some biochemical parameters in guinea-pig. J Pharm Pharmacol 1993;475:784-790.
7. Bigg HF, Cawston TE. All-*trans*-retinoic acid interacts synergistically with basic fibroblast growth factor and epidermal growth factor to stimulate the production of tissue inhibitor of metalloproteinases from fibroblasts. Arch Biochem Biophys 1995;319:74-83.
8. Clark, RAF. In: Clark RAF Editors. The Molecular and Cellular Biology of Wound Repair. New York: Plenum; 1996. p.3-50.
9. Buckley-Sturrock A, Woodward SC, Senior RM, Grifiin GL, Klagsbrun M, Davidson JM. Differential stimulation of collagenase and chemotactic activity in fibroblasts derived from rat wound repair tissue and human skin by growth factors. J

Cell Physiol 1989;138:70-78.
10. McNeil PL, Muthukrishnan L, Warder E, D'Amore PA. Growth factors are released by mechanically wounded endothelial cells. J Cell Biol 1989;109:811-822.
11. Van WW Jr. Wound contraction. Surg Gynecol Obstet 1967;125:131-142.
12. Ramirez AT, Soroff HS, Schwartz MS, Mooty J, Pearson E, Raben MS. Experimental wound healing in man. Surg Gynecol Obstet 1969;128:283-293.
13. Grinnell F. Fibroblasts, myofibroblasts, and wound contraction. J Cell Biol 1994;124:401-404.
14. Lawrence WT, Diegelmann RF. Growth factors in wound healing. Clin Dermatol 1994;12:157-169.
15. Dipietro LA. Wound healing: the role of the macrophage and other immune cells. Shock 1995;4:233-240.
16. DiPietro LA, Burdick M, Low QE, Kunkel SL, Strieter RM. MIP-1alpha as a critical macrophage chemoattractant in murine wound repair. J Clin Invest 1998;101:1693-1698.
17. Kovacs EJ, DiPietro LA. Fibrogenic cytokines and connective tissue production. FASEB J 1994;8:854-861.
18. Beutler BA, Cerami A. Cachectin and tumor necrosis factor as two sides of the same biologic coin. Nature 1986;320:584-588.
19. Vilcek J, Tsujimoto M, Palombella VJ, Kohase M, Le J. Tumor necrosis factor: Receptor binding and mitogenic action in fibroblasts. J Cell Physiol 1987;5:57-61.
20. Dayer J, Beutler B, Cerami A. Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E_2 production by human synovial cells and dermal fibroblasts. J Exp Med

1985;162:2163-2167.
21. Balkwill FR. Tumor necrosis factor. Br Med Bull 1989;45:389-400.
22. Roberts AB, Anzano MD, Lamb LC, Smith JM, Sporn MB. New class of transforming growth factors potentiated by epidermal growth factor: Isolation from nonneoplastic tissues. Proc Nat1 Acad Sci USA 1981;77:5339-5343.
23. Massague J, Cheifetz S, Ignotz RA, Boyd FT. Multiple typebeta transforming growth factors and their receptors. J Cell Physiol 1987;5:43-57.
24. Massague J. The TGF-beta family of growth and differentiation factors. Cell 1987;49:437-438.
25. Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB. Tranforming growth factor-beta in human platelets. J Biol Chem 1983;258:7155-7160.
26. Kehrl JH, Wakefield LM, Roberts AB, Jakowlew S, Alvarez-Mon M, Derynck R, et al. Production of transforming growth factor-beta by human T lymphocytes and its potential role in the regulation of T cell growth. J Exp Med 1986;163:1037-1050.
27. Assoian RK, Fleurdelys BE, Stevenson HC, Miller PJ, Madtes DK, Raines EW, et al. Expression and secretion of type beta transforming growth factor by activated human macrophages. Proc Nat1 Acad Sci USA 1987;84:6020-6024.
28. Sporn MB, Roberts AB, Wakefield LM, de Crombrugghe B. Some recent advances in the chemistry and biology of transforming growth factord. J Cell Biol 1987;105:1039-1045.
29. Lyons RM, Moses HL. Transforming growth factors and the

regulation of cell proliferation. Eur J Biochem 1990;187:467-473
30. Roberts AB, Anzano MA, Wakefield LM, et al. Type β transforming growth factor: a bifunctional regulator of cellular growth. Proc Natl Acad Sci USA 1985;82:119-123.
31. Moses HL, Yang EY, Pietenpol JA, TGF-β stimulation and inhibition of cell proliferation: new mechanistic insights. Cell 1990;63:245-247.
32. Sporn MB, Roberts AB, Wakefield LM, Assoian RK. Transforming growth factor-β: biological function and chemical structure. Science 1986;233:532-534.
33. Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, et al. Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci USA 1986;83:4167-4171.
34. Overall CM, Wrana JL, Sodek J. Transforming growth factor-β regulation of collagenase, 72 kda-progelatinase, TIMP and PAI-1 expression in rat bone cell populations and human fibroblasts. Connect Tissue Res 1989;29:289-294.
35. Edwards DR, Murphy G, Reynolds JJ, Whitham SE, Docherty AJ, Angel P, et al. Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. EMBO J 1987;6:1899-1904.
36. Ignotz Ra, Endo T, Massague J. Regulation of fibronectin and type I collagen mRNA levels by transforming growth factor-β. J Biol Chem 1987;262:6443-6446.
37. Bassols A, Massague J. Transforming growth factor β regulates the expression and structure of extracellular matrix

chondroitin/dermatan sulfate proteoglycans. J Biol Chem 1988;263:3039-3045

38. Fukamizu JH, Grinnell F. Spatial organization of extracellular matrix and fibroblast activity: effects of serum, TGF-β and fibronectin. Exp Cell Res 1990;190:276-282
39. Chavan M, Suzuki T, Recowicz M, Lennarz W. Genetic, biochemical, and morphological evidence for the involvement of N-glycosylation in biosynthesis of the cell wall beta 1,6-glucan of Saccharomyces cerevisiae. Proc Natl Acad Sci USA 2003;100:15381-15386.
40. Czop JK, Puglisi AV, Miorandi DZ, Austen KF. Perturbation of beta-glucan receptors on human neutrophils initiates phagocytosis and leukotriene B4 production. J Immunol 1988;141:3170-3176.
41. Mol PC, Park HM, Mullins JT, Cabib E. A GTP-binding protein regulates the activity of (1→3)-beta-glucan synthase, an enzyme directly involved in yeast cell wall morphogenesis. J Biol Chem. 1994;269:31267-31274.
42. Browder W, Williams D, Lucore P, Pretus H, Jones E, McNamee R. Effect of enhanced macrophage function on early wound healing. Surgery 1988;104:224-230.
43. Portera CA, Love EJ, Memore L, Zhang L, Muller A, Browder W, et al. Effect of macrophage stimulation on collagen biosynthesis in the healing wound. Am Surg 1997;63:125-131.
44. Wolk M, Danon D. Promotion of wound healing by yeast glucan evaluated in single animals. Med Biol 1985;63:73-80.
45. Delatte SJ, Evans J, Hebra A, Adamson W, Othersen HB, Tagge EP. Effectiveness of beta-glucan collagen for treatment

of partial-thickness burn in children. J Pediatr Surg 2001;36:113-118.
46. Compton R, Williams D, Browder W. The beneficial effect of enhanced macrophage function on early wound healing. Am Surgeon 1996;62:14-18.
47. Wolk M, Danon D. Promotion of wound healing by yeast glucan evaluated on single animals. Lab Anim Sci 1985;35:150-152.
48. Kernodle DS, Gates H, Kaiser AB, Prophylactic anti-infective activity of poly-[1-6]-beta-D-glucopyranosyl-[1-3]-beta-D-glucopyranose glucan in a guinea pig model of staphylococcal wound infection. Antimicrob Agents Chemother 1998;42:545-549.
49. Kaiser AB, Kernodle DS. Synergism between poly-(1-6)-beta-D-glucopyranosyl-[1-3]-beta-D-glucopyranose glucan and cefazolin in prophylaxis of staphylococcal wound infection. Antimicrob Agents Chemother 1998;42:2449-2451.
50. Anders GL, Per L, Christer T. (1→3)-β-D-Glucan stimulates nitric oxide generation and cytokine mRNA expression in macrophages. Environ Toxicol and Pharmacol 1998;5:273-281.
51. Stone BA, Clarke AE. Chemistry and Biology of (1→3)-β-D-glucan. La Trobe University Press 1992.
52. Amitabha B, Lorraine HK, Stefen JS, Chin CH. Impaired wound healing in mice deficient in a matricellular protein SPARC (osteonectin, BM-40). BMC Cell Biol 2001;2:15-23.
53. Flanders KC, Thompson NL, Cissel DS, Van Obberghen-Schilling V, Baker CC, Kass ME, et al. Transforming growth factor-β1: histochemical localization with antibodies to different epitopes. J Cell Biol 1989;108:653-660.

54. Mori T, Okumura M, Matsuura M, Ueno K, Tokura S, Okamoto Y, et al. Effects of chitin and its derivatives on the proliferation and cytokine production of fibroblasts *in vitro*. Biomaterials 1997;18:947-951.
55. Matsui R, Okura N, Osaki K, Konishi J, Ikegami K, Koide M. Histological evaluation of skin reconstruction using artificial dermis. Biomaterials 1996;17:995-1000.

Abstract in Korean

(1→3),(1→6)-β-D-Glucan의 창상 치유에 대한 효과

<지도교수 박 종 철>

연세대학교 대학원 의과학과

손 현 주

베타 글루칸은 다당류의 천연 물질로서, 면역 반응을 증가시키고 특히 창상 치유에 효과가 있는 것으로 알려져 왔다. 베타 글루칸의 창상 치유 효과와 관련하여 직접 또는 간접적인 경로의 두 가지 메커니즘이 제시될 수 있다. 그 첫 번째는 베타 글루칸이 섬유아세포의 표면에 존재하는 수용체를 통하여 세포가 이동하고 증식하도록 직접적으로 신호를 보낸다는 가설이다. 이미 베타 글루칸의 수용체가 포유류의 대식세포와 같은 단핵 세포 외에 사람의 진피 세포에도 적어도 둘 이상의 결합 부위를 갖고 있다는 사실이 보고 된 바 있다. 두 번째 가설은, 창상 이후 처리된 베타 글루칸이 대식세포를 자극하여 몇몇 사이토카인과 성장 인자를 분비하도록 하고, 이들이 섬유아세포가 창상 부위로 이동하고 증식하도록 신호한다는 가설이다. 대식세포로부터 분비되는 사이토카인과 성장 인자 중 TNF-α와 IL-6와 같은 염증 관련 사이토카인이나 TGF-β는 특히 창상 치유 과정에서 섬유아세포의 활동과 밀접한 관련이 있다. 이 연구에서는 (1→3),(1→6) 베타 글루칸이 창상 치유 과정 중 섬유아세포에 미치는 직, 간접적인 영향을 생체 내 그리고 생체 밖 실험을 통하여 평가하고자 하였다.

섬유아세포의 증식과 이동 시험의 결과에서 (1→3),(1→6) 베타 글루칸은 사람의 성인 진피세포의 이동에는 효과를 갖지만, 증식에는

효과를 갖지 않았다. 또한 베타 글루칸은 대식세포를 활성화시켜 TNF-α와 TGF-β1의 분비량을 증가시켰으나, IL-6의 양은 증가시키지 않았다. TNF-α는 섬유아세포의 증식을 활성화시키고 콜라겐 합성을 자극하는 것으로 알려져 있다. 그리고 TGF-β1는 창상치유 과정에서 섬유아세포의 증식 및 이동과 세포외 기질의 생성을 증진시킨다. 따라서 이 결과는 베타 글루칸이 대식세포에 의한 사이토카인의 분비를 증가시킴으로써 창상치유에 영향을 미칠 수 있다고 제시한다. 생체 내 창상 치유 시험의 결과 베타 글루칸을 처리한 창상이 식염수나 콜라겐을 처리한 창상보다 빠른 수축을 보였다. 이러한 결과는 (1→3), (1→6) 베타 글루칸의 적용이 창상 치유를 증진하는데 유용하다는 것을 의미한다.

결론적으로, 이러한 일련의 결과들은 (1→3), (1→6) 베타 글루칸이 직, 간접적인 경로 모두를 통해 섬유아세포를 활성화시킴으로써 창상치유에 효과가 있음을 제시한다.

핵심되는 말 : (1→3),(1→6) 베타 글루칸, 창상 치유, 섬유아세포, 증식, 이동, 대식세포, 사이토카인, 성장 인자