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Effect of β-glucan Originated from *Aureobasidium* on the Dermal Wound Repair in Vitro Model

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Abstract : The objective of the present study is to detect the effect of β-glucan originated from *Aureobasidium* on the proliferation and collagen production in human dermal fibroblast cells with wound repopulation in vitro. The proliferative effects were assessed using a MTT assay as well as cell counts at 24 and 48 hr after treatment. Hydroxyproline was measured as an index of procollagen production with reverse-phase high pressure liquid chromatography. Oncostatin M was used as a reference agent. In glucagon treated group, dose-dependent and significant increase of optical density or fibroblast cell numbers was demonstrated, when compared with those of control from 0.1 mg/ml concentration. In addition, the numbers of cells which had migrated into the wound defects were more significantly and dose-dependently increased than those of non-treated control. However, no meaningful effects on the procollagen production were observed.

Key words **:** β-glucan, Aureobasidium, nude mouse, wound management, infection.

Introduction

Cutaneous wound repair is a complex process, which has evolved to achieve rapid restoration of skin integrity and protective function after injury (41). Sometimes repair proceeds inappropriately leading to either chronic wounds where healing is pathologically slowed (24) or to scarring where there is an exuberant and unpredictable synthesis of the extracellular matrix (2,32). These wound healing pathologies are a significant cause of morbidity and consequently there is substantial interest in agents which may modify the wound-healing process (34).

Fibroblasts are the key cells involving the mechanism of wound healing. They were proliferated and activated in case of wound healing (6,26). Fibroblasts are important sources of cytokines, and mediators that can influence the behavior of adjacent cell types and thereby contribute to the initiation and resolution of airway inflammation. They include pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), tumor necrosis factor alpha and anti-inflammatory cytokines such as macrophage inhibitory protein (10). Fibroblasts also release multiple growth factors such as transforming growth factor beta (TGFβ), which directly influence the deposition and composition of the extracellular matrix (10) and may control multiple processes in wound repair (5). Therefore, it has been regarded that alterations in the proliferation and activation of fibroblasts may influence the progression of wound healing. In the present study, the effects of β-glucan on the proliferation of fibroblast were observed in vitro assay using a MTT assay (34,39).

Collagen is an another key material involving the mechanism of wound healing. A component of optimal healing is regulation of collagen formation. Favorable collagen formation includes both enhanced collagen production for healing connective tissues and diminished formation in vital organs to minimize fibrosis (27). Wound healing involves overlapping steps of inflammation, cell migration and proliferation, neovascularisation, extracellular matrix production and remodeling. Collagen is a major component of extracellular matrix (9,19,39). Therefore, the increase of collagen production may influence the progression of wound healing.

Fibroblast-populated collagen lattice (FPCL) model systems (3) are attempted to address some of these issues (34). Fibroblasts are cultured within a hydrated matrix of type I collagen which undergoes cell mediated re-organization and contraction to result in a tissue like structure which, superficially at least, appears to be similar to dermis. This structure has been used extensively to model different aspects of dermal wound repair (23,44,20). Repopulation of the wound space by fibroblasts from the surrounding dermal tissue is essential for wound repair and is achieved through a combination of fibroblast proliferation and migration (3,11,34).

Oncostatin M (OSM) is a pleiotropic cytokine that was first described as a product of inflammatory cells such as T-lymphocytes, monocyte/macrophages and more recently neutrophils (7,16). In dermal fibroblasts, OSM is an activator of the collagen α2 promoter (1,25) and potently induces collagen and glycosaminoglycan production (17,39). Therefore,

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OSM was used as a reference agent on the proliferation and procollagen effects of fibroblast cells in the present study. The fucoidans, as isolated, seem to be a mixture of L-linked fucose polysaccharides sulphated to different extents (4,42). The fibroblast cell migrate effects of fucoidans was well established in the FPCL model systems (21,20) especially on the fraction 7 (FF 7) (34). Thus FF 7 was used as a reference agent on the wound re-population test using FPCL model systems in the present study.

β-glucan is a fiber-type complex sugar (polysaccharide) derived from the cell wall of baker's yeast, oat, barley fiber, and many medicinal mushrooms, such as maitake. The two primary uses of β-glucan are to enhance the immune system (13) and to lower blood cholesterol levels (3,31). In addition, wound healing effects of β-glucan has been reported (8,12,14,29,36,43). However, the direct effects of β-glucan on the wound healing were not reported yet. In addition, the β-glucan used in the present study is purified from *Aureobasidium pullulans* SM-2001 (half of the dry material is -1,3/1,6-glucans), a UV induced mutant of *A. pullulans* and they showed somewhat different characteristics from other β-glucan derived from other origins (40).

This study, therefore, was carried out to examine the effect of β-glucan originated from *Aureobasidium*, on the proliferation and collagen production human dermal fibroblast cells with wound repopulation in in vitro models. In this model, migrated human dermal fibroblast cell numbers were also counted after test article treatments.

Materials and Methods

Cell lines and maintenances

The normal diploid human fetal dermal Fibroblast cell line (FW20-2) was obtained from National Institute of General Medical Sciences (NIGMS, NJ, USA) and was used between passages 12 and 18. Primary human dermal fibroblasts were established from explant cultures (28). Cells were cultured in T-75 tissue culture flasks with fibroblast culture medium (FCM) containing Dulbecco's modified Eagle's Medium (DMEM Invitrogen, UK) supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich, MO, USA), 2 mM L-glutamine, 100 μg/ml penicillin and gentamycin. Cells were kept in a humidified atmosphere of 5% CO_2 in air at 37°C. Medium was routinely changed every 3 to 4 days.

Preparations and administration of drugs

β-glucan of *A. pullulans* SM-2001 (Glucan Corp., Korea, half of the dry material is -1,3/1,6-glucans), OSM (R&D systems, MN, USA) and FF 7 (Calbiocem, CA, USA) are used in this study. β-glucan (2.5% solution) was diluted in FCM at a concentration 100, 10, 1, 0.1 and 0.01 mg/ml concentration. OSM and FF 7 was dissolved in FCM without FCS at 10 ng/ml and 10 mg/ml concentration, respectively. OSM and FF 7 filter-sterilized through a 0.2 μm filter just immediately before treatment.

Cell proliferation assays

Fibroblasts were seeded in DMEM/10% FCS at a density of 3×10^4 cells per well in 24 well tissue culture plates and allowed to adhere for 24 hr. The cells were quiesced by replacing the medium with serum free DMEM for a further 24 h before being treated with either β-glucan at 100, 10, 1, 0.1 and 0.01 mg/ml or OSM 10 ng/ml at 37°C for 24 or 48 hr. At the end of each incubation period, proliferation was assessed using an MTT assay or cell counts. Culture medium was aspirated and replaced with 100 ml of DMEM and 5 ml of MTT solution (5 mg/ml MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) in phosphate buffered saline (PBS)). Dishes were incubated for 4 hrs at 37°C after 100 ml of extraction buffer (10% w/v sodium dodecyl sulphate, SDS, Sigma-Aldrich) in 0.1 M HCl) was added to each well. The absorption was read at 570 nm on a microtiter plate reader. For cell counts, cells were stained with Trypan blue and the number of viable cells was counted using a Haemocytometer.

Determination of fibroblast procollagen production

Fibroblast procollagen production was assessed in vitro by quantitating hydroxyproline (HYP) using reverse-phase high pressure liquid chromatography (7,9,39). Cells were grown to confluence in 2.4 cm diameter wells in DMEM supplemented with 10% FCS and pre-incubated in DMEM supplemented with 1% FCS, 50 g/ml ascorbic acid (BDH, UK) and 0.2 mM praline (Sigma, MO, USA) at 37°C for 24hrs. The cells were then exposed to 100, 10, 1, 0.1 and 0.01 mg/ml β-glucan or 10 ng/ml OSM in similar 1% FCS supplemented culture medium (n = 15 for each dose: five for cell counts and ten for HYP measurement) for a further 48 hr. For HYP measurement, the cell layer was scraped into the culture medium and the contents of each well were collected. Wells were washed with 1 ml PBS and combined with the original medium. Proteins were precipitated by the addition of ethanol to a final concentration of 67% (v/v) at 48°C overnight. Precipitated proteins were separated from free amino acids by filtration through a 0.45 mm pore-size filter (Millipore, UK) and hydrolyzed in 6N HCl at 110°C for 16 hr. Hydrolysates were mixed with activated charcoal, filtered and HYP quantified using reverse-phase HPLC after derivatization with 7-chloro-4-nitroben-zofuran (Sigma, MO, USA). The HYP content in each sample was determined by comparing peak areas of samples from the chromatogram to those generated from standard solutions. All values obtained were corrected for the amount of HYP measured in the cell layer and culture medium at the onset of the incubation period and for cell number. Procollagen production is expressed as nM of hyp/10^6 cells.

FPCL and wounding

Collagen Extraction Rat-tail type I collagen was purified according to Bell *et al.* (1979) with minor modifications by Rowling *et al.* (1990). Collagen was extracted from the rat

tail tendon by incubation in sterile 17 mM acetic acid with mechanical stirring for 48 hr at 4°C. Undissolved tendon pieces were removed by centrifugation at 3000 × g at 4°C in a SS-34 rotor of a Sorvall centrifuge for 60 min. Sterile 0.1M NaOH was used to adjust the pH of the collagen solution to pH 7 causing precipitation of the collagen. The precipitated collagen was collected by centrifugation at 1000 × g at 4°C in a GSA rotor of a Sorvall centrifuge for 20 min. The type I collagen pellet was transferred to sterile 17 mM acetic acid where it re-dissolved upon stirring at 4°C for 48 hr.

FPCLs were fabricated according to previous reports (3,38). To fabricate FPCLs into one 12-well cluster tissue culture plate the following mixture was prepared on ice: 4 ml of 2 × DMEM (to make 100 ml combine 20 ml of 10 × DMEM, 10 ml 7.5% NaHCO3, 2 ml L-glutamine, 2 ml NEAA (Invitrogen, UK) with 66 ml sterile qH2O), 1 ml FCS, 1 ml FCM containing 1×10^4 fibroblasts, 3 ml of 5 mg/ml collagen solution, and 1 ml NaOH. After mixing, 800 ml were pipetted into each well. The dishes were maintained at 37°C in a 5% CO_2/95% air atmosphere for 1 hr to allow collagen polymerization. After polymerization 2 ml of FCM was added to each well. Lattices were detached from the plastic substratum by ringing gently with a 23-gauge hypodermic needle and allowed to contract for 5-7 days until they reached approximately 10% of their starting diameter. FPCLs were wounded by 3 mm punch biopsy and the wounded FPCL transferred to a cellular collagen gel fabricated identically to the method for FPCLs above but substituting 1 ml of FCM for the fibroblast suspension. Ten microliters of un-polymerised collagen was used to adhere the FPCL to the underlying collagen lattice. Defect repopulation was then measured by counting the number of cells which had migrated from the cut edge of the lattice at 3 days post wounding and treatment of test articles. Results are expressed as a mean of cell counts in ten identical photographic fields from ten replicated wounds

Statistical analyses

All data was calculated as mean ± S. D. (n = 10). Statistical analyses were conducted using Mann-Whitney U-Wilcoxon Rank Sum W test (MW test) with SPSS for Windows (Release 6.1.3., SPSS Inc., USA). The inhibition rate compared to that of vehicle control was calculated to help the understanding of the efficacy of test materials on differences between vehicle control and test groups

Results

Effects of β-glucan on the proliferation of dermal fibroblasts

Significant ($p < 0.01$) increases of OD and fibroblast cell numbers were detected in OSM at 24 and 48 hr of incubation compared to those of control. These OD and fibroblast cell numbers were also significant ($p < 0.01$) and dose-dependent increased from 0.1 mg/ml of β-glucan treatment. In 0.01 mg/ml of β-glucan, significant ($p < 0.05$) increase was restricted to the cell numbers at 48 hr, non-significant increases were observed at 24 and 48 hr of incubation (Table 1).

The OD at 24 hr of incubation showed % changes vs vehicle control as 86.78, 85.83, 77.11, 57.63, 38.56 and 11.17% in OSM, β-glucan 100, 10, 1, 0.1 and 0.01 mg/ml-treated groups, respectively. The OD at 48 hr of incubation showed % changes vs vehicle control as 75.18, 74.10, 70.95, 56.11, 51.62 and 3.41% in OSM, β-glucan 100, 10, 1, 0.1 and 0.01 mg/ml-treated groups, respectively.

The cell numbers at 24 hr of incubation showed % changes vs vehicle control as 102.95, 92.30, 77.53, 67.41, 53.27 and 19.83% in OSM, β-glucan 100, 10, 1, 0.1 and 0.01 mg/ml-treated groups, respectively. The cell numbers at 48 hr of incubation showed % changes vs vehicle control as 66.21, 65.22, 57.17, 43.79, 37.34 and 12.89% in OSM, β-glucan 100, 10, 1, 0.1 and 0.01 mg/ml-treated groups, respectively.

The effect of β-glucan on procollagen production

No meaningful changes on the procollagen production of fibroblast were detected in all tested groups compared to that of vehicle control except for OSM, in which a significant

Table 1. Effects of β-glucan on the fibroblast proliferation

Concentration	Optical Density (Arbitrary units)		Absolute cell number ($\times 10^6$)	
	24hrs[1)]	48hrs	24hrs[1)]	48hrs
Control	0.73 ± 0.08	0.95 ± 0.10	2.32 ± 0.19	3.04 ± 0.31
β-glucan				
100 mg/ml	1.36 ± 0.27*	1.82 ± 0.37*	4.03 ± 0.47*	5.03 ± 0.36*
10 mg/ml	1.30 ± 0.20*	1.68 ± 0.29*	3.96 ± 0.44*	4.78 ± 0.31*
1 mg/ml	1.16 ± 0.31*	1.59 ± 0.18*	3.62 ± 0.42*	4.37 ± 0.37*
0.1 mg/ml	1.02 ± 0.31*	1.45 ± 0.24*	3.51 ± 0.38*	4.18 ± 0.42*
0.01 mg/ml	0.82 ± 0.11	1.14 ± 0.26	2.40 ± 0.21	3.43 ± 0.39**
Oncostatin M				
10 ng/ml	1.37 ± 0.19*	1.92 ± 0.16*	4.06 ± 0.39*	5.06 ± 0.44*

n = 10; (Mean ± S.D.); 1) incubation times; * $p < 0.01$ and ** $p < 0.05$ compared to that of vehicle control by MW test.

Table 2. Effects of β-glucan on the procollagen production of fibroblast and fibroblast repopulation of the wound defect

Concentration	Procollagen (nM Hyp/10^6 cells)	Migrated cells (numbers/defect)
Control	8.45 ± 1.27	128.00 ± 8.41
β-glucan		
100 mg/ml	8.47 ± 1.23	191.20 ± 23.94*
10 mg/ml	8.42 ± 1.09	170.80 ± 22.76*
1 mg/ml	8.67 ± 1.28	161.70 ± 12.18*
0.1 mg/ml	8.41 ± 0.95	139.30 ± 17.49
0.01 mg/ml	8.39 ± 1.09	133.00 ± 18.48
Oncostatin M		
1 ng/ml	12.78 ± 2.47*	
Fucoidan Fraction 7		
10 mg/ml		169.60 ± 15.21*

n = 10; (Mean ± S.D.); *$p < 0.01$ compared to that of vehicle control by MW test.

($p < 0.01$) increase of procollagen production was detected compared to that of control (Table 2).

The procollagen production of fibroblasts showed % changes vs vehicle control as 51.18, 0.20, −0.44, 2.56, −0.53 and −0.71% in OSM, β-glucan 100, 10, 1, 0.1 and 0.01 mg/ml-treated groups, respectively.

The effect of β-glucan on the In Vitro wound repopulation

Significant ($p < 0.01$) increases of fibroblasts migrated from the cut edge of the wound in FPCL into the defect were detected in all tested groups compared to that of control except for 0.1 and 0.01 mg/ml of β-glucan-treated groups, where non-significant increase of migrated cells were also observed (Table 2).

The migrated fibroblast numbers showed % changes vs vehicle control as 32.50, 49.77, 33.44, 26.33, 8.83 and 3.91% in OSM, β-glucan 100, 10, 1, 0.1 and 0.01 mg/ml-treated groups, respectively.

Discussion

Although, the accelerating wound healing effects of β-glucans have been studied (14,32,29), the evidences that other polysaccharides accelerate wound healing were also have been evaluated (8,36,12). However, the direct effects of β-glucan on the wound healing were not reported yet. In addition, the β-glucan used in the present study is purified from *Aureobasidium pullulans* SM-2001 (half of the dry material is -1,3/1,6-glucans) and they showed somewhat different characteristics from other β-glucan derived from other origins (40). As a results of β-glucan treatments, dose-dependent and significant increase of OD or fibroblast cell numbers were demonstrated compared to those of control. In addition, the numbers of cells which had migrated into the wound defects were significantly and dose-dependently increased compared with non-treated control cultures. However, no meaningful effects on the procollagen production were observed. These data suggest that β-glucan has properties which may be beneficial in the treatment of wound healing.

The proliferation effects of fibroblasts of β-glucans has little attention, even if the accelerating wound healing effects of β-glucans have been reported (14,43,29) and they showed proliferating effect against human keratocytes (16) and mouse spleen cells (45). In the present study, β-glucan proliferated the human dermal fibroblast at least from 0.1 mg/ml of concentration, but the involved mechanisms is not understood. However, the other polysaccharide, fucoidan showed the proliferating effects on the fibroblasts via cytokine, TGF-β (34). Therefore, the possible mechanisms of β-glucan on the proliferation of fibroblasts are also considered that it act mediated the cytokine and TGF-β. It is seems to be need that the studies should be tested to solve the possible action mechanism of β-glucan on the proliferation effects of fibroblasts. Anyway, fibroblasts are the key cells involving the mechanism of wound healing and therefore, it has been regarded that alterations in the proliferation and activation of fibroblasts may influence the progression of wound healing. β-glucan showed the proliferation effects on the fibroblasts in the present study.

Wound healing involves overlapping steps of inflammation, cell migration and proliferation, neovascularization, extracellular matrix production and remodeling, and the collagen is major component of extracellular matrix (19). The increase of collagen production may influence the progression of wound healing. However, β-glucan do not showed any effects on the pro-collagen production of dermal fibroblast in the present study upto 100 mg/ml of concentration, quite differed from the results of other β-glucans, Wei *et al*. (43) reported that glucan stimulates human dermal fibroblast collagen biosynthesis through a nuclear factor-1 dependent mechanism. These differences are considered as originated from different source of tested β-glucans.

Wound healing is a highly complex yet elegantly co-ordinated process. Medical science would like to be able to intervene when pathology disturbs normal healing. Like many biological phenomena the study of wound healing has benefited from the use of in vivo and in vitro models which have attempted to identify, isolate and simplify some of the process associated with tissue repair. The fibroblast populated collagen lattice system is a useful model to isolate and study aspects of cutaneous wound healing. Further enhancements such as the addition of other cell types allow the re-epithelialization (35) and angiogenesis (15). We have used the FPCL model system to examine the rate of fibroblast repopulation of a wound space. Within this FPCL model there are low rates of fibroblast proliferation prior to wounding (3,31). Wounding activates cell proliferation and migration from the collagen matrix into the wounded defect via migration across

the underlying collagen lattice (22,42). Repopulation of the wound space by fibroblasts from the surrounding dermal tissue is essential for wound repair and is achieved through a combination of fibroblast proliferation and migration (11). In the present study, the effects of β-glucan on the repopulation of dermal fibroblast were observed in vitro using FPCL model system as previously (3,34). β-glucan facilitated the migration of fibroblast into the defects like FF 7. It is considered as that β-glucan accelerates the wound healing. However, it is thought that the in vivo studies for wound healing test of β-glucan is necessary to observe the exact efficacy of β-glucan.

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생체외 모델에서 아우레오바지디움 유래 베타 글루칸이 피부창상 치유에 미치는 영향

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요 약 : 본 연구의 목적은 in vitro 실험에서 human dermal fibroblast cell을 이용한 섬유아세포 증식과 콜라겐 생성을 통한 전층피부창상치유에 대한 효과를 알아보는 것이다. In vitro 실험에서, 베타글루칸 및 양성대조물질 oncostatin M을 사용하여 처치 후 24 또는 48시간 이후 MTT assay와 세포수 관찰을 통해 섬유모세포 증식효과에 대해 평가하였다. Procollagen 생산에 대한 효과를 평가하기 위해 베타글루칸 및 양성대조물질 oncostatin M 처치 48시간 후 hydroxyproline을 HPLC를 이용하여 정량하여 평가하였다. 창상재구축에 대한 효과를 평가하기 위해 베타글루칸 및 양성대조물질 fucoidan fraction 7을 FPCL 모델을 이용하여 평가하였다. 베타글루칸 처치군은 음성대조군 대비 0.1 mg/ml 이상의 농도에서 용량의존적이고 유의적인 OD 또는 섬유아세포수의 증가가 관찰되었다. 게다가 베타글루칸 처치군은 창상면으로 유주된 세포수가 음성대조군 대비 1 mg/ml 이상의 농도에서 용량의존적이고 유의적인 증가가 관찰되었다. 그러나 베타글루칸 처치군은 음성대조군 대비 모든 용량에서 procollagen 생산에 대한 유의적인 변화가 관찰되지 않았다.

주요어 : β-glucan, Aureobasidium, nude mouse, 창상관리, 감염.