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Development of fungal mycelia as skin substitutes: Effects on wound healing and fibroblast

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Abstract

In this study, Sacchachitin membrane, prepared from the residue of the fruiting body of *Ganoderma tsugae*, was estimated for its effects on wound healing and the proliferation and migration of fibroblast cells. Two mirror-image wounds were made on the back of female guinea pigs by dissecting a $1.5 \times 1.5\ cm^2$ skin surface of full thickness. Sacchachitin membrane was placed randomly on one of the wounds and gauze or Beschitin® on the other. Changes in the wound area were measured and photographed after a predetermined amount of time postoperatively. Histological examination of the wound and surrounding tissue was also performed to reveal any interaction of tissue with the dressing. The results showed that the wound area covered with Sacchachitin membrane was statistically smaller than that covering with gauze on day 10, whereas there was no significant difference in the wound size compared to that with Beschitin®. Fibroblast cells from the dermis layer of guinea pigs were used. The number of fibroblast cells were counted on the predetermined days in the culture suspended with or without 0.01% w/v dressing materials. By layering on DMEM plates, the number of fibroblast cells migrating across the center line or outside of the central hole were counted after five days. All the results indicated that both 0.01% w/v of Sacchachitin and chitin significantly enhanced the proliferation and migration of fibroblast cells.

Keywords: *Ganoderma tsugae*; Fungal mycelia; Wound healing; Fibroblast; Proliferation; Migration

1. Introduction

Wound healing is defined as the restoration of the continuity of living tissue and is an integrated response of several cell types to injury. It involves platelet aggregation and blood clotting, the formation of fibrin, an inflammatory response, alteration in the ground substance, endothelial and capillary proliferation and surface covering, regeneration of certain cell types, variable contracture and remodeling [1]. Healing is not complete until the disrupted surfaces are firmly knit by collagen. Generally, the use of a skin substitute to provide an environment conducive to healing is necessary [2]. In an endeavor to develop an ideal skin substitute, the performance of Sacchachitin membrane, prepared from the residue of the fruiting body of the medicinal fungus, *Ganoderma tsugae*, as an effective skin prosthesis has been examined [3]. This study evaluated the effectiveness of Sacchachitin membrane in the management of excised wounds in guinea pigs and compared its performance with gauze and Beschitin®.

A variety of wound models have been employed to study the wound healing process. The techniques that have been employed involve morphological examination of the wound size, histological examination of biopsied tissue samples, the detection of collagen content, the number of cells in the new connective tissue and epithelial layers [2], and the measurement of some biochemical parameters [1]. In conjunction with the area measuring technique and histological examination, evaluation of the effect of Sacchachitin on the proliferation and migration of fibroblasts in culture was included.

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2. Materials and methods

2.1. Materials

The residue of the fruiting body of *Ganoderma tsugae* was collected after hot water extraction twice and was a generous gift from a factory in Natuao, Taiwan. Beschitin-W®, made from crab chitin, is a commercial product of Morihita Resere Co. (Japan). Ketamine HCl and xylazine were supplied by Sigma Co. (St. Louis, MO, USA). Female guinea pigs, weighing from 380 to 480 g and aged from 8–10 weeks, were purchased from the Animal Center, National Taiwan University. Analytical-grade reagents were obtained from Merck Co. (Germany). Deacetylated Sacchachitin was obtained by dissolving Sacchachitin in 45% NaOH with heating. The resulting solution was dialyzed with tap water for two days and then with distilled water for one day resulting in a solution with a neutral pH. This solution was then freeze-dried to obtain deacetylated Sacchachitin. β-Glucan was the alkaline-soluble fraction of the polysaccharides obtained by treating the residue of the fruiting body of *Ganoderma tsugae* with 1 N NaOH at 90°C for 4 h.

2.2. Preparation of Sacchachitin membrane

The purification of fibers to form Sacchachitin membrane was followed a similar procedure that reported in the previous paper [3], except that the treatment with 1 N NaOH was conducted at 90°C for 4 h. The fibers with lengths in the range of 10–50 μm were then collected and dispersed in deionized water to form a suspension. The suspension was then filtered. The membrane formed on the filter paper was then freeze-dried (EYELA, model FD-5N) to obtain a porous membrane with a diameter of 7 cm and thickness of 0.1–0.2 mm for the following studies. The chemical constituents of the final product was determined to be 40% *N*-acetyl-D-glucosamine and 60% β-1,3-D-glucan. The membranes were autoclaved and kept under aseptic conditions until use.

2.3. Wound healing studies

Prior to the study, guinea pigs were anesthetized separately with Ketamine ($35\ mg\ kg^{-1}$) and xylazine ($5\ mg\ kg^{-1}$) by abdominal injection. The dorsal and abdominal hairs of the guinea pigs were removed with an electric razor. The method proposed by Kaufman was followed to prepare wounds [4]. Two equal mirror-image areas were marked on the dorsal area of the guinea pigs 1 cm apart from the spinal cord and in between the 12th rib and iliosaacral joint. Two pieces of full thickness skin, each with a surface area of about $1.5 \times 1.5\ cm^2$, were excised. The method of excision was similar to that reported by Smahel [5]. The depth of the excised area was as deep as the panniculus carnosus. After cleansing off the blood residues with gauze and 0.9% saline solution, one of the lesions was randomly chosen and covered with an equal size of cotton gauze or Beschitin for comparison. The other side was covered with Sacchachitin membrane as prepared above. Both dressings were hydrated with 0.9% saline solution to promote the adhesion of the dressings to the wound surface. Treated guinea pigs were placed in individual cages with an air-filtering device in a temperature range between 22–28°C with humidity control.

After surgery, the area of the wound was measured on the 5th, 10th, 15th, and 20th days. Fresh dressings were replaced at the same time. A modified method of Nangia to calculate the wound area was employed [2]. Generally, the outline of the wound area was marked on a transparent paper and then transcribed to another piece of ordinary paper. A hand-held scanner (Proscan Gray) was used to capture the image and data were stored as a monochromic BMP file. With proper adjustment, a computer program written with Visual Basic was employed to calculate the wound area so obtained. A total of 15 guinea pigs were included in this study. The statistical significance of any difference was analyzed by a paired Student's *t*-test.

2.4. Histological analysis

On days 5, 10, 15, and 20, one of the guinea pigs from the above study was killed. Lesions with the surrounding tissue were excised in a deep-V shape. Specimens were then fixed in 36% formalin for 2–3 h and then cut into two halves to promote the infiltration of formalin into the tissue. Specimens were dehydrated with a tissue auto-treatment device (Sakura, RH-12E) and embedded in paraffin with a Paraffin Dispenser (Shadon, Lipshow). Sections of appropriate thickness (about 5 μm) were sliced (Sakura, IVS-400) and stained with haematoxylin and eosin. Entellan (Merck) was used to seal the specimen before examination using a Hitachi model S-2400 SEM.

2.5. Implantation

The implantation was according to the modified method proposed by Peluso [6]. All animals were anaesthetized in the same way as described above. A 1 cm square of autoclave-sterilized Sacchachitin was implanted into the lesion between the subcutaneous tissue and muscular membrane on the dorsal area of the guinea pigs 1 cm away from the spinal cord and in between the 12th rib and iliosaacral joint. On the opposite side, either gauze or Beschitin was implanted as a control. Both lesions were then closed with 3-O nylon sutures. Guinea pigs were kept in individual cages and the sutures removed on day seven. On day 14, the implants were excised together with the surrounding tissue. The specimens

were then fixed in 36% formaldehyde for 2–3 h and embedded in paraffin. Sections of appropriate thickness were then sliced and examined using a Hitachi model S-2400 SEM.

2.6. Scanning electronic microscopy (SEM) examinations

The Sacchachitin membrane samples harvested on day 9 from lesions were washed three times for 15 min with 0.1% cacodylate buffer solution (pH 7.4) containing 7% sucrose. The samples were then fixed in cacodylate buffer solution containing 1% OsO_4 for 1.5 h. After that, the samples were washed again according to the same procedure as described above. The specimens were dehydrated by immersion in a series of aqueous solutions of increasing alcohol content, followed by critical point drying using liquid CO_2 as the transfer medium (Hitachi, HCP-2). Dried samples were then loaded on aluminum studs and coated with gold for 3 min at 8 mA under a pressure of 0.1 Torr (Hitachi, IB2). The samples were scanned and examined using a Hitachi model S-2400 SEM. One control sample was prepared by simply immersing another Sacchachitin membrane in phosphate buffer for nine days and a second control sample was untreated Sacchachitin.

Part of the Sacchachitin membrane obtained from the wound healing studies was treated with 10% NaOH to remove blood clots and was then cleaned with distilled water until the pH was neutral. Membranes were pressed between two glass slides and treated with 0.5% periodic acid for 5 min. After that, they were washed with water several times and then incubated in Schiff's solution for 15 min. After washing with water for 10 min, a purple color could be visualized on these membranes. A control sample of Sacchachitin stored in phosphate buffer solution was also prepared.

2.7. Preparation of fibroblast cells

Fibroblast cells were acquired by the method of primary culture. A piece of skin was isolated from the dorsal area of a guinea pig and sterilized in iodine–alcohol solution for 30 s and then in 70% alcohol for 15 s. It was then cut into several pieces each with a surface area of about 2 mm^2. After attaching to a tissue culture dish for 20 min, 10 ml of culture medium (DMEM with 15% v/v fetal bovine serum, 1% v/v streptomycin) was added followed by storage for a couple of days in an incubator (Sanyo, MC0175) controlled at 37°C and with 5% CO_2. Fibroblast cells were harvested simply by removal of skin specimens [7].

2.8. Proliferation studies of fibroblast cells

Fibroblast cells at a concentration of about 3×10^4 cells ml^{-1} was placed in the DMEM medium in a petridish 35 mm in diameter. After incubation for 24 h, the medium was changed with fresh medium containing various materials at a concentration of 0.01% w/v. The materials tested included Sacchachitin powder, deacetylated Sacchachitin, chitin powder, *N*-acetyl-D-glucosamine, and β-1,3-D-glucan. On a predetermined day, 0.25% Trypsin-EDTA was added to detach the cells. About 50 μl of cell suspension was sampled and mixed with an equal volume of 0.5% Trypan blue. The total number of live cells was then counted using a hemocytometer under light microscopy [8]. A growth curve was plotted for each material added to the medium. The results were an average of replicated samples. At the same time of sampling, 70% of the medium was replaced with fresh medium. On day 9, another cell suspension was sampled and fixed with 70% aqueous alcohol solution followed by the addition of 300 μl of propidium iodide solution (50 μg ml^{-1}). The number of cells in the solution was counted for 5 min by a flowcytometer (FACScan, Becton Dickson). Data was analyzed with LYSIS 2 software. The effects of two different concentrations (0.05 and 0.1% w/v) of *N*-acetyl-D-glucosamine, deacetylated Sacchachitin and β-1,3-D-glucan, were also examined.

2.9. Migration studies of fibroblast cells

Fibroblast cells (3×10^5 cells ml^{-1}) were incubated in the DMEM medium containing 0.2% FCS for 72 h in a petridish. Aphidicolin (0.5 mg ml^{-1}) was added and the incubation continued for another 24 h. About 1.2×10^5 of these cells were incubated in a petridish of 35 mm in diameter until the cells were grown fully over the whole dish area. A cell lifter was used to mark a cross line in the center of the dish and those cells on one side were scratched off. The remaining cells on the other side were cleaned twice with phosphate buffer solution. Then, a medium containing 0.01% w/v of various materials (chitin, deacetylated Sacchachitin, and β-glucan) was added. Incubation was done at 37°C with 5% CO_2. The extent of migration was estimated by counting the number of cells growing across the central line at six different points each within an area of 600×600 μm^2. Differences among different materials were analyzed based on the Student's *t*-test with $P < 0.05$ [8–10].

Another way of estimating the migration of fibroblast cells was as followed. The test materials suspended in 1% agarose medium were placed in a petridish and a layer consisting of a precipitate of the test material later formed at the bottom of the petridish. A hole was punched with a Pasteur pipette in the center of the solidified agarose medium. About 1000–1500 cells were placed into the hole, 15 min later, the cells precipitated to the bottom of the hole. The hole was then covered with DMEM medium and incubated at 37°C with 5% CO_2 for a certain period of time. From the center of the hole, the cells that migrated along the interface between the layers of

agarose and of the test material were counted in six different areas of equal size but randomly chosen [11–12]. The Student's *t*-test was used to analyze any significant difference among the materials tested.

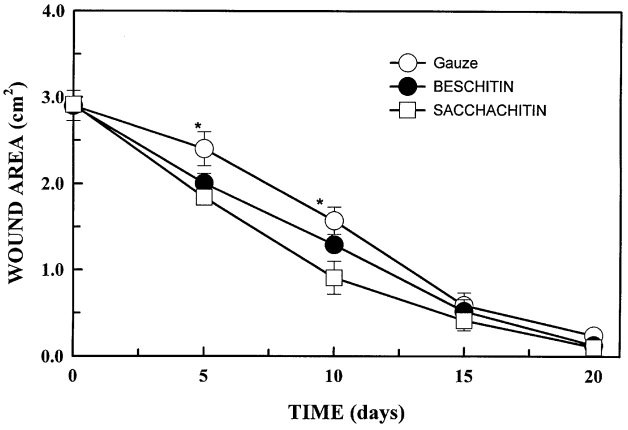

Fig. 1. Comparison of wound area changes at different time intervals when using Sacchachitin, Beschitin and gauze to cover the wound. (*: a paired *t*-test of significant difference with $P < 0.05$).

3. Results and discussion

Changes in the wound area covered either with Sacchachitin, Beschitin or gauze were estimated on days 5, 10, 15, and 20. The average change in the wound area is shown in Fig. 1 and demonstrates that the wound area covered with Sacchachitin measured at the above mentioned time was smaller than that covered with gauze. However, the difference between the area covered with Sacchachitin and that with Beschitin was not significant. Histological examination of wound tissue showed that new cells were apparently formed on day 10 in wounds covered with both Sacchachitin or Beschitin. On day 20, the differentiation of the hair follicles was also observed. However, the new cells only appeared on day 15 in wounds covered with gauze with shrinking of the wound area being observed also. Histological examination further revealed that the infiltration of numerous polymorphonuclear leukocytes into the wound area covered with Sacchachitin and Beschitin, whereas only a few monocytes were found to have infiltrated the wound area covered with gauze. These results are shown in Figs. 2–4.

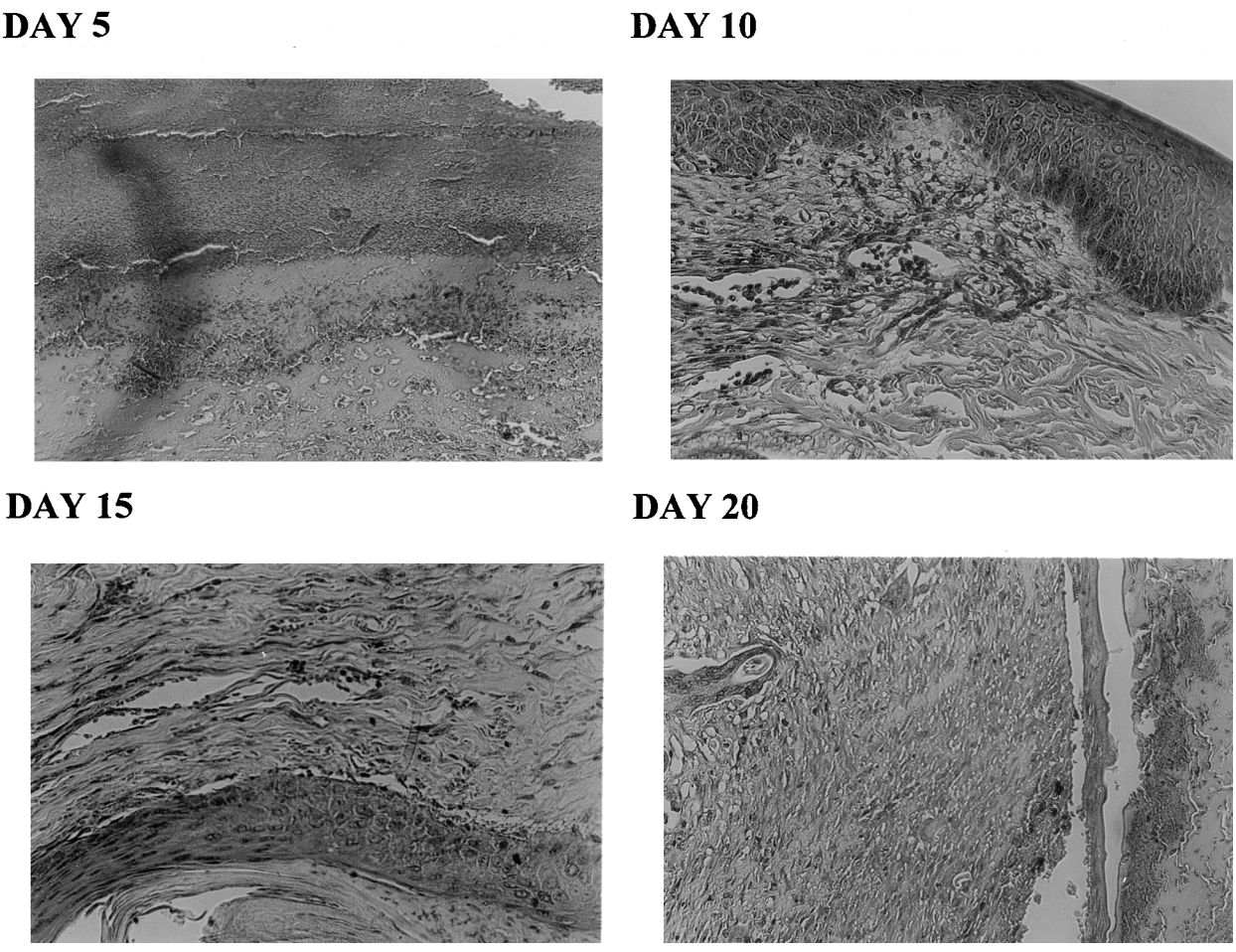

Fig. 2. Photomicrographs of the wound area and its surrounding tissue covered with Sacchachitin membrane at different time intervals.

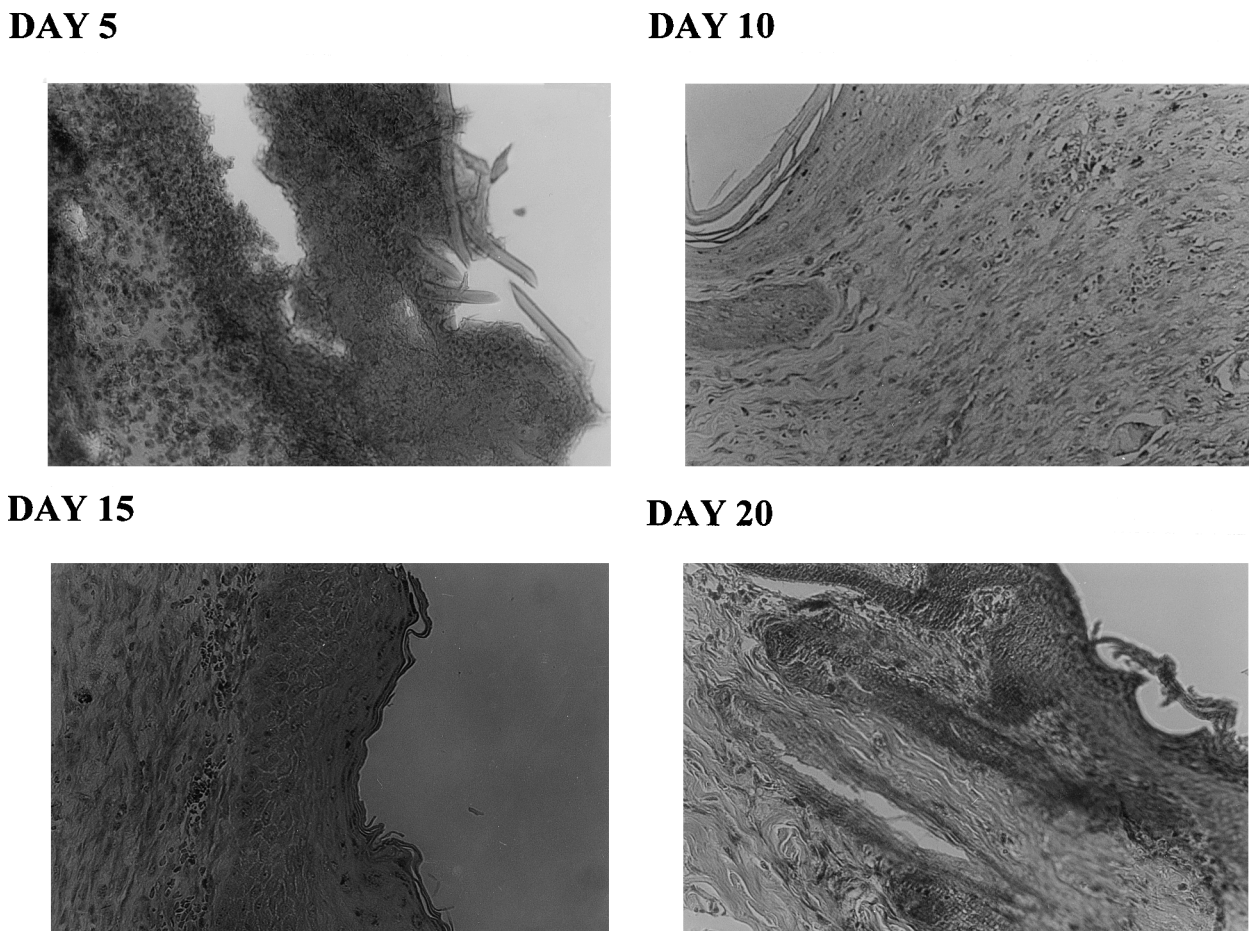

Fig. 3. Photomicrographs of the wound area and its surrounding tissue covered with Beschitin membrane at different time intervals.

The accumulation of polymorphonuclear leukocytes, such as neutrophils and eosinophils, in wound areas covered with either Sacchachitin or Beschitin is a sign of an acute inflammatory reaction. However, no bacterial infection was observed, indicating that both materials had a chemotactic effect on the inflammatory cells. On the contrary, the infiltration of a large amount of monocytes, i.e. lymphocytes and macrophages, into the wound area covered with gauze is attributed to a type IV allergic reaction. This is a type of rejection phenomenon involving the activation of these chronic inflammatory cells causing necrosis of the wound tissue or the appearance of blisters on the skin. In 1976, it was reported that a mild acute inflammatory reaction of a wound caused by infection in the earlier stage of trauma was able to accelerate healing of the wound and to increase the ability to tolerate the tension after healing as well [13]. It was also found that numerous polymorphonulcear leukocytes with some macrophages, which are able to secrete cell cytokines or growth factors, had accumulated in the vicinity of the wound. Possible reasons proposed for the acceleration of wound healing by the author was attributed to the action of cell cytokines or growth factors on promoting the differentiation of granuloma granulation tissue in the wound area [13]. This would explain the acceleration of wound healing by Sacchachitin membrane and Beschitin.

When Sacchachitin membrane isolated from the wound area was examined, it appeared that the structure of the mycelia hypha were destroyed and would not stain with PAS (Fig. 5A). On the other hand, the untreated Sacchachitin membranes and that immersed in phosphate buffer solution (Fig. 5B) showed no signs of damage to the structure of the mycelia hypha and they could be stained with PAS. Thus, it appears that the constituents of Sacchachitin membrane that can be stained by PAS were eliminated during the wound healing process.

It has been known that chitin is hydrolyzable by lysozyme [14]. Since chitin is a portion of the Sacchachitin structural unit with some soluble polysaccharides, it appears likely that substances released by the infiltration of body fluid caused dissolution of the chitin to promote wound healing. SEM examination confirmed that the structure of the mycelia hypha was disintegrated and would not stain with PAS. Generally, polysaccharides with 1,6-linkage can be stained by PAS. The

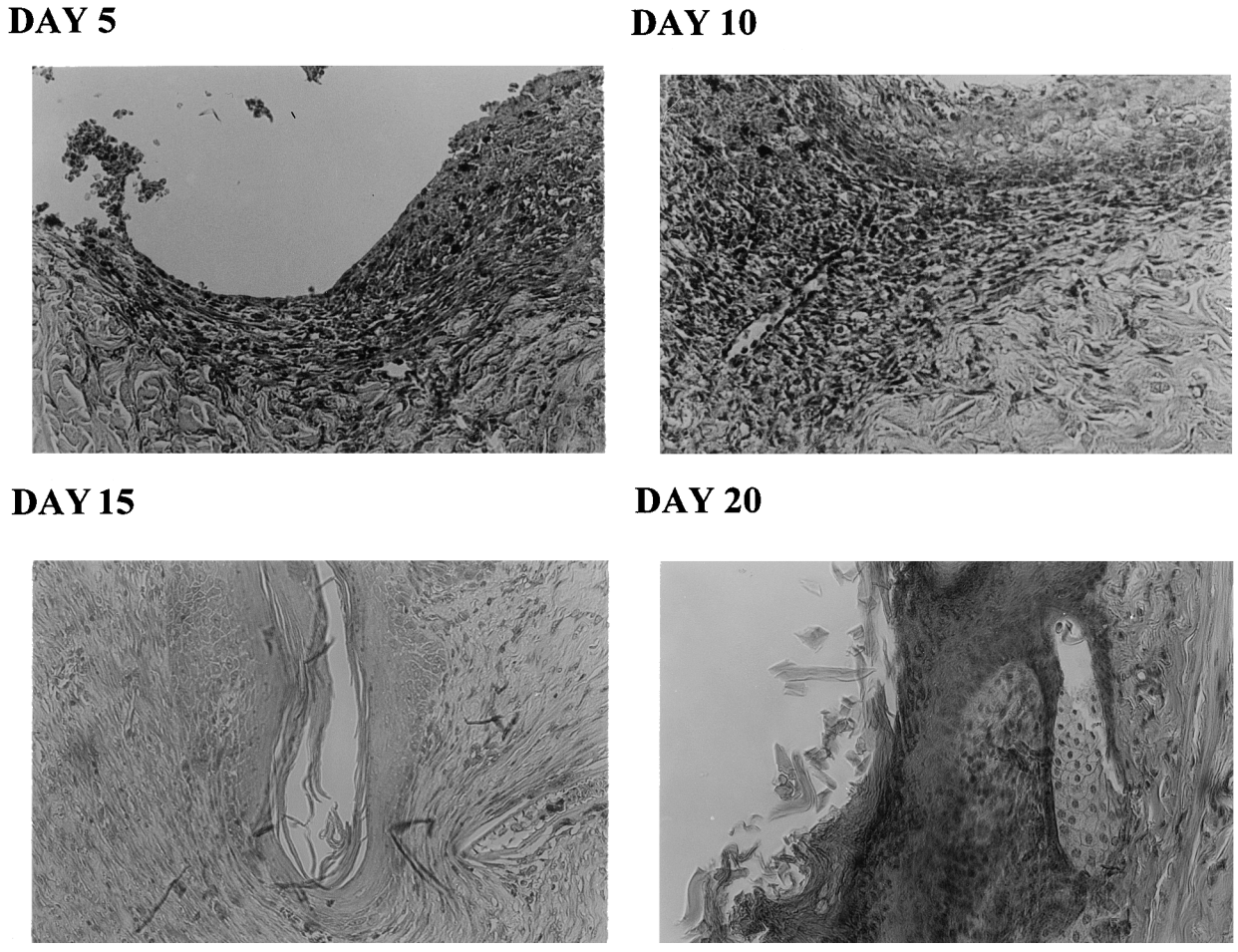

Fig. 4. Photomicrographs of the wound area and its surrounding tissue covered with gauze at different time intervals.

disappearance of the stainability of Sacchachitin membranes covering the wound indicates that this type of polysaccharide had disintegrated and been released. It is possible that the release of these substances can play an important role in the promotion of wound healing.

In order to determine which substances are responsible for the acceleration of wound healing, fibroblast cells, the main component of the dermis, were employed to compare the effect of several materials on their proliferation and migration. The results in Fig. 6 show that the proliferation of fibroblast cells was observed with both of 0.01% w/v Sacchachitin and Beschitin on day 6. Nevertheless, no significant effect on the proliferation of fibroblast cells was noticed until day 9 for 0.01% w/v *N*-acetyl-D-glucosamine and deacetylated Sacchachitin. It demonstrated that there is no difference for 0.01% w/v β-glucan compared to the control. The same results were obtained no matter which method was used for counting the number of cells.

The effect of different concentrations of these soluble materials on the proliferation of fibroblast cells was further examined and the results are plotted in Fig. 7. At a concentration of 0.05% w/v, *N*-acetyl-D-glucosamine and deacetylated Sacchachitin were able to promote the proliferation of fibroblast cells by day 6, whereas this did not occur until day nine for β-glucan (Fig. 7a). When the concentration of β-glucan was increased to 0.1% w/v, the proliferation of fibroblast cells occurred by day three, whereas the proliferation was inhibited with *N*-acetyl-D-glucosamine and deacetylated Sacchachitin (Fig. 7b).

In 1994, Chung et al. [15] reported that chitin isolated from the cell wall of some molds at a concentration of 0.01% w/v was shown to promote the proliferation of fibroblast cells. The same results were observed in this study. In 1987 [16] and 1992 [17], β-glucan was reported to be capable of promoting cell proliferation and macrophage function. However, the promotion of the proliferation of fibroblast cells was not observed until the concentration of β-glucan was increased above 0.05% w/v in this study. On the contrary, increasing the concentration of *N*-acetyl-D-glucosamine and deacetylated Sacchachitin to 0.1% w/v resulted in inhibition of fibroblast cell proliferation. In 1994, the same phenomenon was observed by Richard et al. [15]. Promotion of the

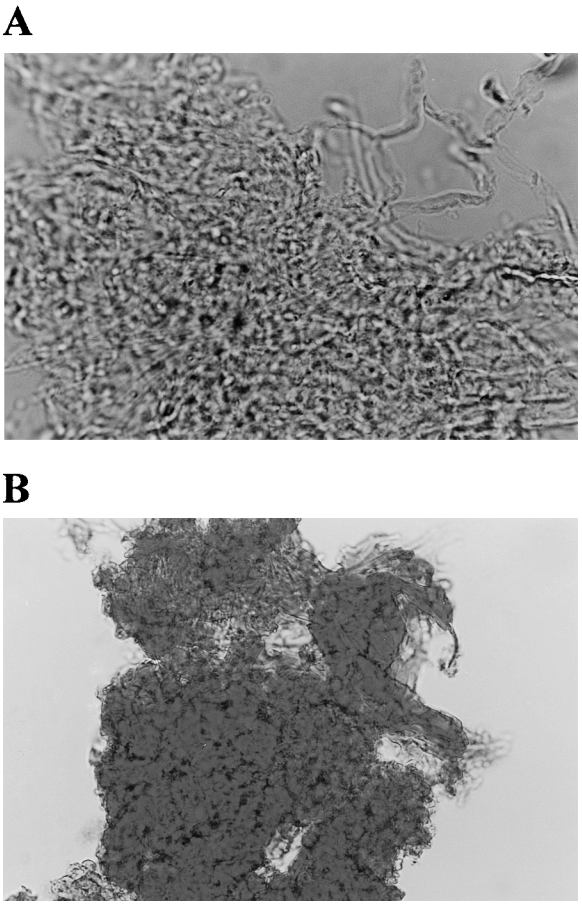

Fig. 5. Photomicrographs of Sacchachitin membranes covering the wound (A) immersed in phosphate buffer solution (B) stained by PAS.

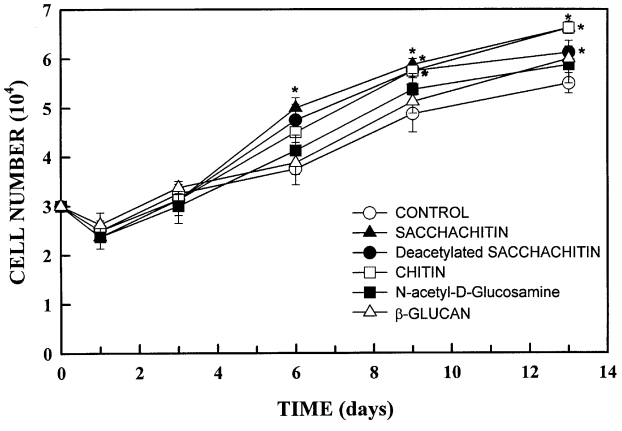

Fig. 6. Comparison of the proliferation of fibroblast cells induced by the addition of several different materials at a concentration of 0.01% w/v in the incubation medium. (*: a paired t-test of significant difference with $P < 0.05$).

proliferation of fibroblast cells has been attributed to the positive charge carried by chitin increasing the adhesion to the cell surface at lower concentrations. However, the interference of cell membrane function by the adhesion of

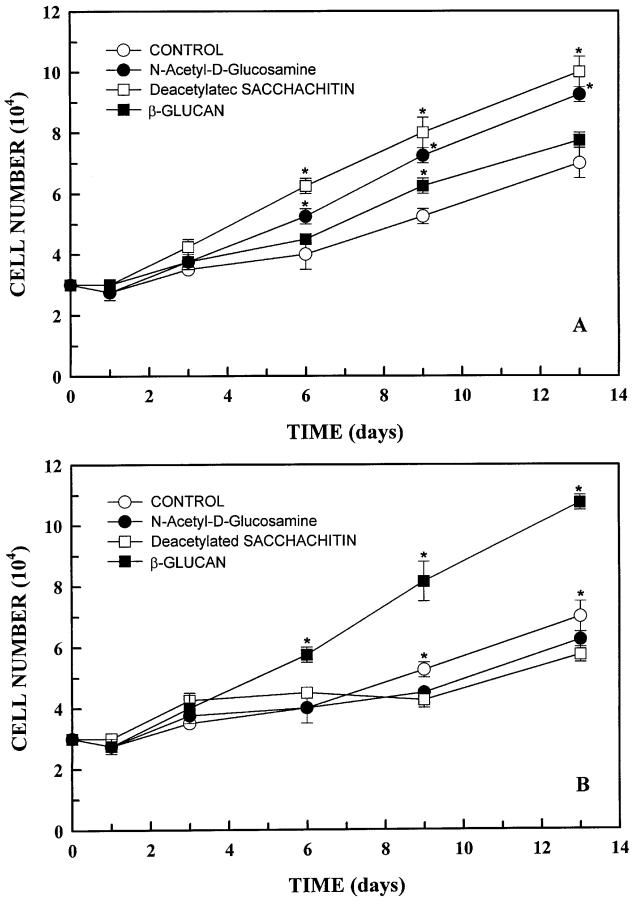

Fig. 7. Comparison of the proliferation of fibroblast cells induced by the addition of soluble materials at a concentration of (A) 0.05% or (B) 0.1% w/v in the incubation medium. (*: a paired t-test of significant difference with $P < 0.05$).

chitin leading to the death of cells was observed at higher concentrations.

Fibroblast cells controlled at the G_0/G_1 phase by aphidicolin were employed in two ways to estimate the extent of migration induced by different materials at a constant concentration of 0.01% w/v. In estimating the extent of migration across the central line in this study, it was found that Sacchachitin and chitin were able to promote extensive migration of fibroblast cells after the 3rd day. No difference in β-glucan from the control was noted. These results are shown in Fig. 8A. Similar results were observed in the migration study as estimated by counting the number of cells growing along the interface between the layers of agarose and the materials from the center hole in the medium (Fig. 8B). The only exception was that the cells migrating along the chitin layer were found to extract their peusdopodium and tended to die. Both results demonstrated that Sacchachitin membrane was able to promote the migration of fibroblast cells and act as a guide for cell growth.

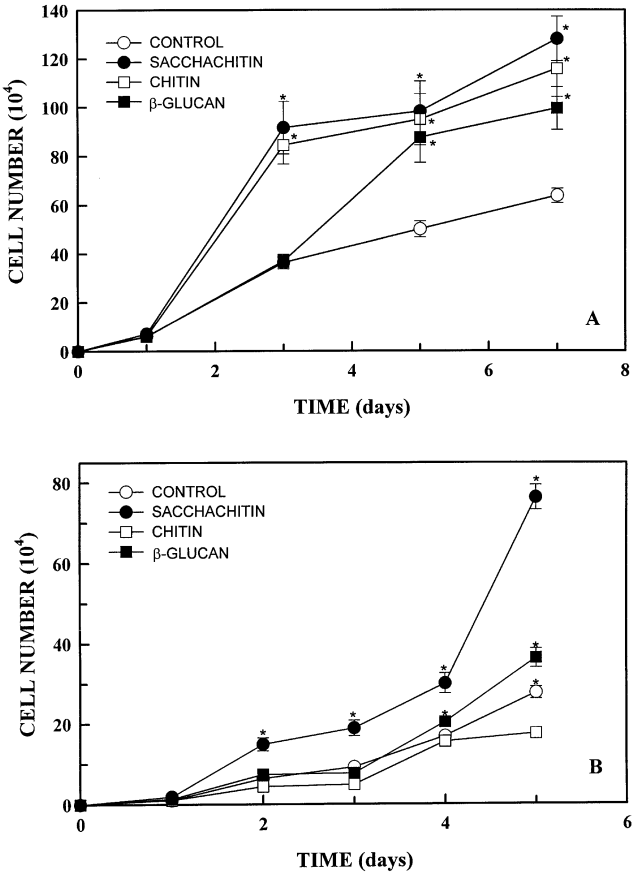

Fig. 8. Comparison of the extent of fibroblast cell migration (A) across the center line of the medium or (B) outside the central hole induced by the addition of soluble materials at a concentration of 0.1% w/v in the incubation medium. (*: a paired t-test of significant difference with $P < 0.05$).

4. Conclusions

Sacchachitin membrane is able to promote wound healing by inducing cell proliferation. A mild acute inflammatory reaction attracted a large number of polymorphonuclear leukocytes and some macrophages to clean away debris and blood clots. Also the secretion of cell cytokines and growth factors by these cells provided an excellent environment for wound healing. The migration of fibroblast cells, which was promoted by Sacchachitin, also plays another important role in the acceleration of wound healing. Optimally, the performance of Sacchachitin membrane as a skin substitute is comparable to the commercial product Beschitin. Further studies on the physical characteristics of the membrane, such as its air permeability and mechanical strength, would be valuable.

Acknowledgements

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