Development of fungal mycelia as a skin substitute: Characterization of keratinocyte proliferation and matrix metalloproteinase expression during improvement in the wound-healing process

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Abstract: SACCHACHITIN membranes, prepared from the waste residue of the fruiting body of *Ganoderma taugae,* were used in our previous study to enhance skin wound healing in animal models. In the present study, the effects of the membrane on the growth of keratinocytes and the activity of matrix metalloproteinases (MMPs), as well as on the healing of skin wounds in humans, were estimated. Fresh human foreskin was employed as the source of the keratinocyte culture, and a modified keratinocyte-SFM medium supplemented with 0.2 ng/mL of recombinant epidermal growth factor and 30 µg/mL bovine pituitary extract was used to enhance the successful growth of keratinocytes under an atmosphere of 5% CO_2, at 37°C. The results indicated that 0.01% SACCHACHITIN enhanced the proliferation of keratinocytes in the culture on the fourth and fifth days, and cells showed neither morphological alteration nor disordered proliferation. This evidence clearly indicated that SACCHACHITIN was not cytotoxic to and was safe for the growth of keratinocytes. Thus, SACCHACHITIN might play a positive role in the proliferation and differentiation of keratinocytes around wounds and in accelerated wound healing of epidermal tissue. In addition, microscopic observations during the growth of keratinocytes showed that normal proliferation and differentiation took place along the margin of the SACCHACHITIN membrane. This indicates that SACCHACHITIN is possibly cytocompatible with keratinocytes. Electrophoretic analysis and inhibition tests for the binding effect of SACCHACHITIN on MMPs showed that SACCHACHITIN reduced MMPs in extracellular matrix degradation and facilitated establishment of an extracellular matrix around wounds; these effects resulted in rapid wound healing. SACCHACHITIN was used as a skin dressing for patients who had skin chronicle ulcer, which had not healed for over 7 months. Preliminary clinical observations showed that the wound improved and began to heal. An analysis of MMPs by ELISA in tissue of the wound indicated a significant decrease in MMP levels. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 72A: 220–227, 2005

Key words: *Ganoderma tsugae;* fungal mycelia; wound healing; MMP; keratinocyte; SACCHACHITIN

INTRODUCTION

The skin wound-healing process is known to be regulated by cells/extracellular matrix (ECM) interactions and by cytokines and/or growth factors. In skin cells, injury is likely to initiate a series of changes in gene expression that mediate downstream events required for tissue repair, including cell proliferation, migration, and remodeling of the ECM.[1] Wound healing is often segregated into three stages: (1) inflammation (early and late); (2) re-epithelialization and granulation tissue formation; and (3) matrix formation and remodeling.[2] During wound healing, proteinases, including serine proteinases such as the urokinase–plasminogen activator and plasmin, and matrix metalloproteinases (MMPs) are implicated in epidermal repair.[3–5] These proteinases primarily facilitate keratinocyte movement by remodeling extracellular matrix proteins. In addition, proteinases modulate intracellular signaling, secretion, bioactivation, and

stability of cytokinases and growth factors important for epidermal healing.[6–10]

Matrix metalloproteinases (MMPs) were first demonstrated to be involved in wound healing in guinea pigs.[11] MMPs are subdivided into five main classes by structure and substrate specificity: collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, and MMP-11), membrane-type MMPs (MT-MMPs, MMP-14, MMP-15, MMP-16, and MMP-17), and an undefined set.[12] It has been shown that MMP-1 is closely related to epithelial migration and is essential for wound re-epithelialization,[13–14] that MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are important during angiogenesis[15] and prolonged matrix remodeling,[14] and that MMP-3 is crucial for normal wound contraction.[16]

The current use of wound-dressing materials is based mostly on empirical knowledge rather than on an actual understanding of the healing process. Balassa showed that pulverized chitin aids wound healing based on observations relating to the mechanical resistance of scar tissue.[17] It was claimed that a process for facilitating wound healing indicated that chitin acted as a wound-healing accelerator.[18–19] It was also reported that chitin is physiologically soluble as a consequence of the effect of lysozyme, suggesting that N-acetylglucosamine is important for the orientation and crosslinking of collagen, and that uridine diphosphate N-acetylglucosamine is a key compound in the biosynthesis of hyaluronic acid. Widra[20] only described associations of chitosan and keratin or collagen, but discovered no biological or histological effect of chitosan on tissue components. Yano[21] demonstrated that chitin increases traction resistance compared to controls, but did not increase the quantity of collagen in the healing tissues. Malette and Quigley,[22] when using chitosan acetate salt to heal wounds, speculated that if fibrin clot formation is avoided, fibroblasts will not be stimulated, and cells can replicate the lost tissue and reduce the thickness of scar tissue. Ricardo[18] delineated the high susceptibility of a chitosan derivative (5-methylpyrrolidone) to depolymerization by lysozyme when applied *in vivo* to a wound, providing an efficient way to stimulate macrophages and spleen cells, and favoring the ordered deposition of collagen, while providing glucosamine and N-acetylglucosamine monomers to the biosynthetic route of hyaluronic acid and glycosaminoglycans.

In an endeavor to develop an ideal skin substitute, the performance of SACCHACHITIN membranes, prepared from residues of the fruiting body of the medicinal fungus, *Ganoderma tsugae,* as an effective skin prosthesis has been examined.[23] That previous study evaluated the effectiveness of SACCHACHITIN membranes in the management of excised wounds in guinea pigs and compared their performance with gauze and BESCHITIN®. However, the underlying mechanisms responsible for the accelerated wound healing by SACCHACHITIN were not disclosed. SACCHACHITIN membranes were demonstrated to accelerate wound healing by inducing cell proliferation in another study.[24] With a covering of SACCHACHITIN, it was found that a mild acute inflammatory reaction attracted a large number of polymorphonuclear leukocytes and some macrophages to clean away debris and blood clots during the healing process. It was also speculated that the secretion of cell cytokines and growth factors by these cells provided an excellent environment for wound healing. The migration of fibroblast cells, which is promoted by SACCHACHITIN, was also suspected to play another important role in the acceleration of wound healing. However, all those facts are not sufficient to demonstrate the effect of SACCHACHITIN on re-epithelialization of keratinocytes and how it improves the healing of chronic wounds, which is a result of imbalance between MMPs and TIMPs (tissue inhibitors of metalloproteinases). In this study, the influence of SACCHACHITIN membranes on the proliferation of keratinocytes and the activity of MMPs was characterized.

MATERIALS AND METHODS

Reagents

The residue of the fruiting body of *Ganoderma tsugae* was collected after two hot-water extractions and was a generous gift from a factory in Nantou, Taiwan. Ketamine HCl and xylazine were supplied by Sigma (St. Louis, MO, USA). Female guinea pigs, weighing 380 to 480 g and aged 8 to 10 weeks, were purchased from the Animal Center, National Taiwan University. Analytical-grade reagents were obtained from Merck (Germany). Deacetylated SACCHACHITIN was obtained by dissolving SACCHACHITIN in 45% NaOH with heating. The resulting solution was dialyzed with tap water for 2 days and then with distilled water for 1 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. SACCHACHITIN was cut into pieces and suspended in deionized phosphate-buffered solution (D-PBS). This suspension was ultrasonicated until homogeneous and subsequently steam sterilized. A SACCHACHITIN stock suspension was obtained by dispensing the resulting suspension into medium to a concentration of 0.5% w/v. An MMP-8 substrate solution was prepared by dissolving MMP-8 substrate powder in 0.1% trifluoroacetic acid, and then this was diluted with stock solution containing 50 mM tricine, 200 mM NaCl, 10 mM CaCl$_2$, 0.05% BRIJ®, and 50 μM ZnSO$_4$, pH 7.5, to a desired concentration.

Preparation of SACCHACHITIN membranes

The purification of fibers to form SACCHACHITIN membranes followed a procedure similar to that reported in a 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 to 50 μm were collected and dispersed in deionized water to form a suspension, which was then filtered. The membrane formed on the nylon filter cloth was freeze-dried (EYELA, model FD-5N) to obtain a porous membrane with a diameter of 7 cm and thickness of 0.1 to 0.2 mm for the following studies. The chemical constituents of the final product were 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.

Isolation and primary culture of human keratinocytes

Human foreskin samples were obtained from Taipei Medical University Hospital. Isolation of human keratinocytes followed the procedure described by Ham and colleagues.[6] Primary cells were seeded into T-75 flasks at a cell density of 3×10^5 cells/mL. Cultures were incubated at 37°C in an atmosphere containing 5% CO_2.

Assessment of keratinocyte proliferation

Third-passage human keratinocytes were seeded into petri dishes (40 mm) at a cell density of 2×10^4 cells/mL to which the SACCHACHITIN stock suspension (0.5% w/v in medium) had been added at a concentration of 0.01% w/v. At each predetermined time point, three culture samples were treated with 0.05% of a trypsin–EDTA solution to dissociate cells for determination of cell numbers. Thirty microliters of each cell suspension was sampled with the addition of an equivalent volume of 0.5% trypan blue. A hemocytometer was used to count the cell numbers under light microscopy, and a growth curve was then drawn for a 5-day period. The morphology of keratinocyte cells was examined by light microscopy to compare the cytotoxicity of the SACCHACHITIN suspension with the controls. A cell culture with no added SACCHACHIYTIN stock suspension was used as the control.

In vitro activity measurements of MMP-1, MMP-8, and MMP-9

MMP (5.6 mU/mg for MMP-8, 2.07 mU/mg for MMP-9, and 0.45 mU/mg for MMP-1) was added to an MMP substrate solution (1.2 μM for MMP-8 and 0.5 μM for MMP-9 and MMP-1). A mixture was prepared in a 10-mm cuvette by thoroughly mixing the above solution with a SACCHACHITIN stock solution to produce concentrations of 0.0025%, 0.001%, 0.01%, or 0.025%. The resulting activity of MMP was monitored by measuring the fluorescence at respective excitation and emission wavelengths (MMP-8, 280 and 360 nm; MMP-9 and MMP-1, 365 and 450 nm). A standard sample with no addition of SACCHACHITIN was used as the control for comparison. The same procedure for measuring the activity of MMP was repeated by replacing SACCHACHITIN with N-acetylglucosamine at the same four concentrations and with chitin at the two concentrations of 0.001% and 0.0025% only.

In vitro binding of MMP-1, MMP-8, and MMP-9

After thoroughly mixing seven separate sample solutions, including MMP substrate, MMP + MMP substrate, MMP + MMP substrate + 0.1% SACCHACHITIN, MMP + 0.1% SACCHACHITIN, MMP substrate + 0.1% SACCHA-CHITIN, MMP, and 0.1% SACCHACHITIN with a half volume of sampling buffer (2×), mixtures were heated to 100°C for 5 min. Fifteen microliters of the resulting solution was loaded and subjected to SDS-PAGE on 12% SDS gel along with 8 μL of protein standard at room temperature and 130 V until the tracking dye reached the bottom of the gel. After electrophoresis, gels were stained with Coomassie brilliant blue R-250, destained, and dried for visualization.

Activity measurement and quantitation of MMP in a human chronic wound

This research project was approved by the University Institutional Review Board. Informed consent was obtained for all procedures. Two patients with impaired wound healing for more than 7 months were recruited. Wounds were cleaned with saline solution and then divided into two areas. One area was covered with a SACCHACHITIN membrane and gauze was placed on the top, while the other area was covered with only gauze. At predetermined time intervals, the healing of the wounds was observed and recorded, and tissues from both areas were sampled for analysis.

Wound tissue was homogenized in buffer solution (50 mM Tris-HCl, 1 mM monothioglycerol, 1 mM phenylmethane sulfonylfluoride, pH 7.4) and centrifuged (2000 g for 10 min) to obtain the supernatant. The protein content in the supernatant was measured based on the Bradford protein assay method (Bio-Rad reagent) using BSA as a standard.

The activity of MMP in the supernatant was measured as follows: 100 μL of sample (containing 100 μg of protein) and standard were incubated in microtiter wells precoated with anti-MMP antibody at 4°C overnight. After washing and aspiration of unbound components, 50 μL of 1 mM p-aminophenylmercuric acetate (APMA) was added and incubated at 37°C for 2 h to activate the bound pro-MMP. Equal volumes of pro-detection enzyme (modified urokinase) and chromogenic substrate (S-2444 peptide) were then added and incubated at 37°C for another 2 h. The absorbance at 450 nm was then read.

Amounts of MMP-8 and MMP-9 in the supernatant extracted from wound tissue were quantified as follows: 100-μL aliquots of sample (containing 50 μg protein) and
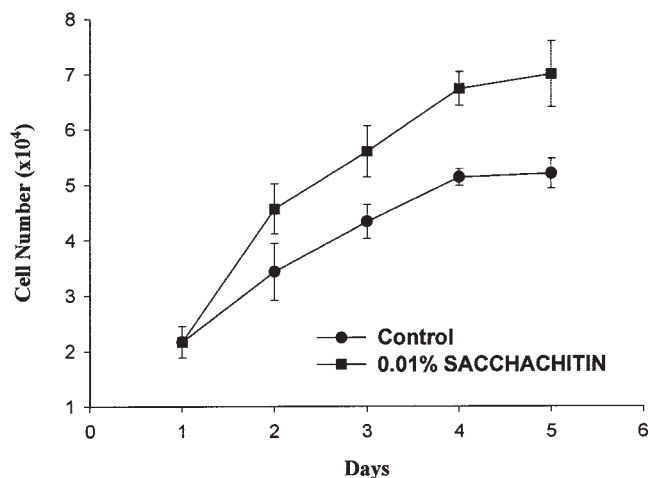
Figure 1. Growth curve of keratinocytes in medium containing 0.01% SACCHACHITIN (values represent the mean $\pm$ SD, $n = 3$).

standard were incubated in microtiter wells precoated with anti-MMP antibody at room temperature for 1 h. After washing and aspiration of the unbound components, 100 μL of anti-MMP horseradish peroxidase was added and incubated at room temperature for 2 h. After that, free substrate was cleaned off, and 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added immediately. Twenty minutes after incubation at room temperature, 100 μL of 1 M sulfuric acid was added to stop the reaction, and the absorbance at 450 nm was read.

RESULTS

The influence of SACCHACHITIN on the growth of human keratinocytes (HKs) was examined. Figure 1 demonstrates that the promotion of proliferation of HK was slight with the presence of 0.01% SACCHA-CHITIN on the second day but became profound on days 4 and 5. The morphology of HK cells cultured for 72 (A) and 168 h (B) is shown in Figure 2. No obvious change in morphology was observed compared to those cultured with no SACCHACHITIN. It was concluded that SACCHACHITIN could accelerate wound healing by promoting proliferation of HKs and by having low cytotoxicity and being biocompatible with HK cells.

The influences of SACCHACHITIN and its possible monomer, N-acetylglucosamine, on the activity of MMP-1 were examined. Figure 3(A) indicates that the extent of inhibition of MMP-1 activity increased with an increasing added amount of both substances. In comparison with the control, a statistically significant inhibition of activity was observed when the concentration of N-acetylglucosamine exceeded 0.001%, whereas inhibition was observed when the concentration exceeded 0.01% for SACCHACHITIN. This indi-

cates that inhibition of the activity of MMP-1 was less effective when using SACCHACHITIN than when using N-acetylglucosamine.

Figure 3(B) illustrates that the inhibition on the activity of MMP-8 increased with an increasing added amount of SACCHACHITIN, and statistically significant inhibition ($p < 0.01$) was observed when the added amount exceeded 0.001% in comparison with the control. On the contrary, inhibition of the activity of MMP-8 by N-acetylglucosamine decreased with an increasing added amount and did not show statistically significant inhibition at the highest added amount (0.025%). Nevertheless, both added amounts of 0.001% and 0.0025% of chitin isolated from crab shell demonstrated significant inhibition on the activity of MMP-8 similar to that of SACCHACHITIN. Inhibition of the activity of MMP-8 for all three substances at these two low added amounts of 0.001% and 0.0025% was determined not to significantly differ.
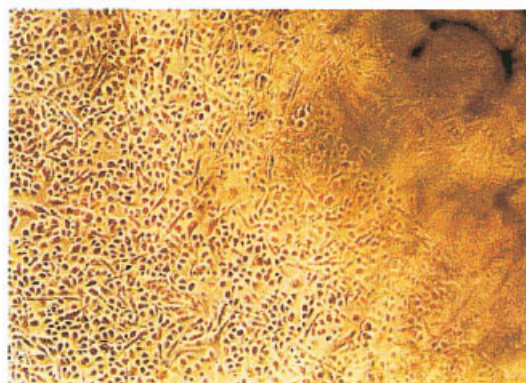
(A)

(B)

Figure 2. Morphology of keratinocytes co-cultured in the presence of SACCHACHITIN (0.01%) for 72 (A) and 168 h (B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
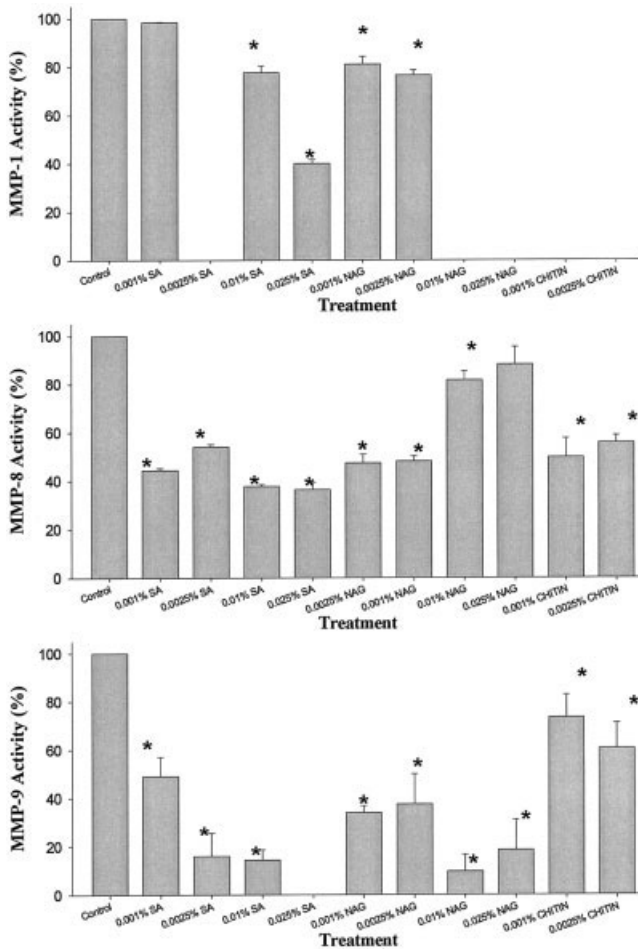
Figure 3. MMP (A, MMP1; B, MMP-8; C, MMP-9) activities in the presence of various concentrations of SACCHA-CHITIN (SA) or *N*-acetyl-glucosamine (NAG) (values represent the mean $\pm$ SD, $n = 3$; *$p < 0.01$ vs the control).

Figure 3(C) shows that inhibition of the activity of MMP-9 increased with an increasing added amount of SACCHACHITIN and *N*-acetylglucosamine. This inhibiting effect was less significant at the two added amounts of 0.01% and 0.025% for chitin in comparison with the control. Because significant inhibition of the activity of MMP-9 was observed at a concentration of as low as 0.001% for SACCHACHITIN, we concluded that SACCHACHITIN was the most effective for inhibiting MMP-9 activity among these three substances.

The binding effect of SACCHACHITIN on MMP was examined by determining the migration of MMP proteins during electrophoresis. Figure 4 displays densitographs of electrophoretic migration of the three MMP proteins, respectively. The relative densitograms for the corresponding bands at the molecular weights of 44 kDa for MMP-1, of 58 kDa for MMP-8, and of 92 kDa for MMP-9 after electrophoresis were selected for comparison of the extent of free MMP available for migration. All three demonstrated that

the extent of free MMP available for electrophoretic migration decreased in the presence of 0.01% SAC-CHACHITIN. This effect seemed to be more profound for MMP-1 and MMP-9. This demonstrates that the binding effect of MMP by SACCHACHITIN might inhibit the activity of the three MMP proteins.

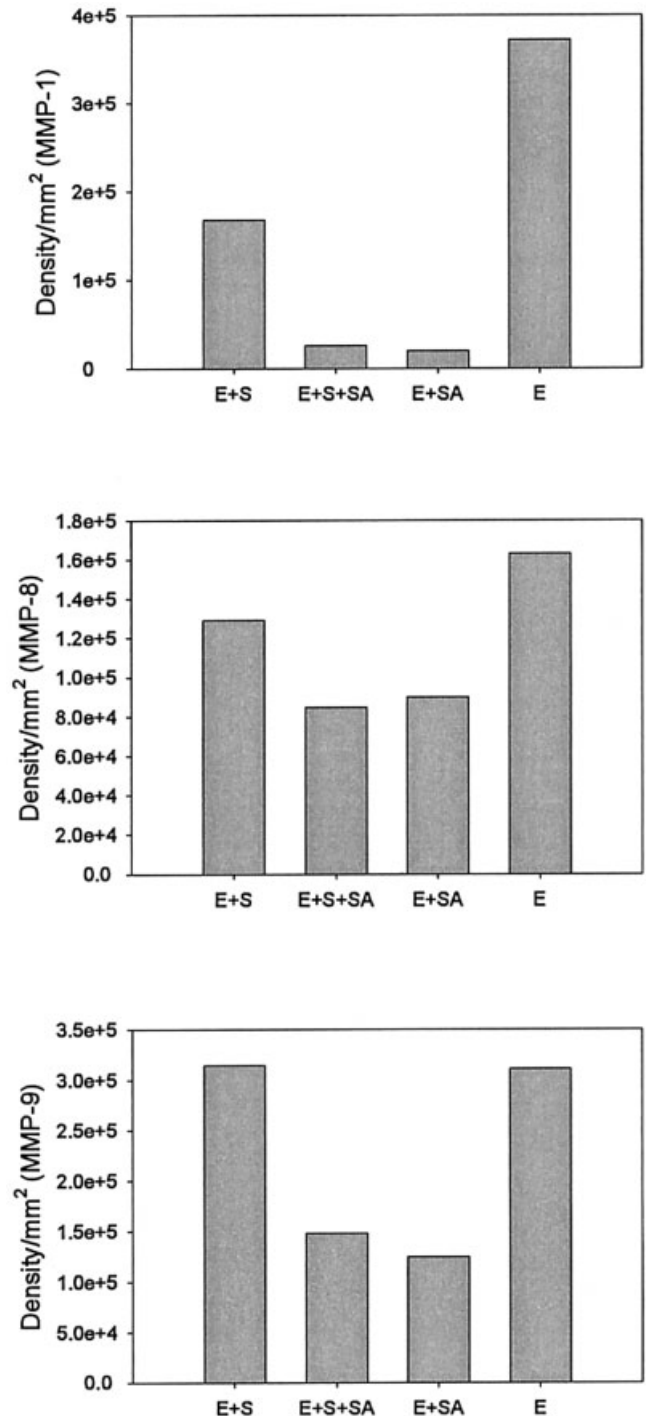Figure 5(A) shows the accelerated healing of the

Figure 4. Binding effects of SACCHACHITIN on MMPs (A, MMP-1; B, MMP-8; C, MMP-9). E, MMP enzymes; S, MMP substrates; SA, SACCHACHITIN.
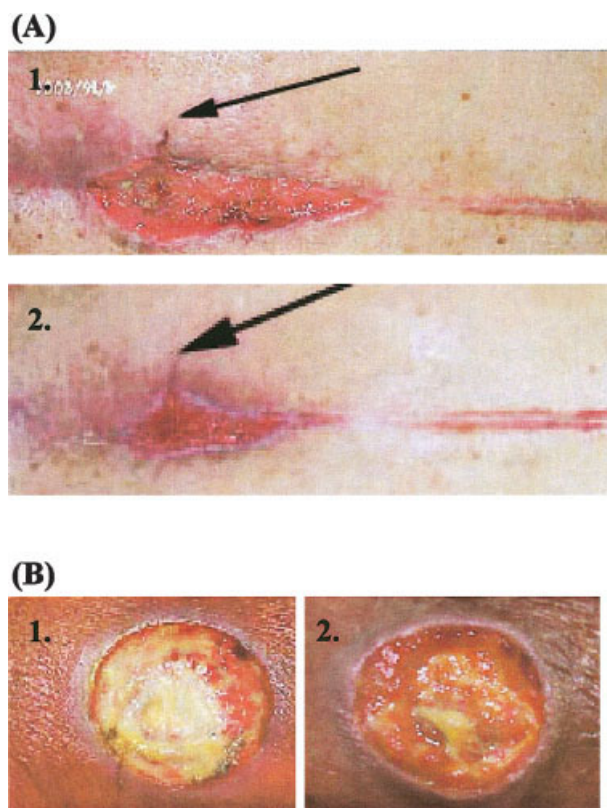
Figure 5. The healing process of chronic wounds (two cases, A and B) covered with SACCHACHITIN membranes. (1) before, (2) after 10 days of treatment. [Color figure can be viewed in the online issue, which is available at www. interscience.wiley.com.]

infected wound covered with SACCHACHITIN. This wound area originally showed impaired healing for almost 7 months after treatment (by surgery). The debris was then cleaned off by treatment with antibiotics, and the wound was divided into two areas: one of which was covered with SACCHACHITIN and the other covered with gauze. Two weeks after treatment, healing in the area covered with SACCHACHITIN had improved in comparison to that covered with gauze in terms of shrinkage of the depth and surface area of the wound. Figure 5(B) shows another wound which exhibited impaired healing for up to a year after treatment. However, the half of this area covered with SACCHACHITIN for 1 month showed that granular tissue in the peripheral area of the wound had proliferated in an accelerated rate in comparison to that covered with gauze.

Quantitative analysis of protein levels of MMP-1, MMP-8, and MMP-9 and their activities in these skin wound tissues covered with either SACCHACHITIN or gauze was performed, and sole results for MMP-9 are illustrated in Figure 6. It demonstrates that the content and activity of MMP-1 in those skin wound tissues covered with SACCHACHITIN did not significantly differ from those covered with gauze either

before or after treatments (data not shown). The content of MMP-9 in two cases of skin wound treatment with SACCHACHITIN decreased after treatment compared to that before treatment. There was only one case of skin wound tissue covered with gauze showing a significant change in the content of MMP-9 after treatment. The activities of MMP-9 in skin wound tissue before and after either treatment showed no obvious changes. The content and activity of MMP-8 in skin wound tissue for both treatments were also inconsistent in each individual case (data not shown).

DISCUSSION

SACCHACHITIN is obtained from the residue of the fruiting body of *Ganoderma tsugae* after extraction of chemical constituents. It contains 40% chitin and 60% β-glucan. In 1970, Prudden and colleagues[25] confirmed that chitin is able to accelerate wound healing. Previously, it was demonstrated that SACCHACHITIN membranes are able to promote wound healing by inducing cell proliferation. Histological examination of skin wound tissue treated with SACCHACHITIN also revealed that 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, plays another important role in the acceleration of wound healing as well.

During the process of wound healing, proliferation and differentiation of keratinocytes are an indispensable factor. Cell culture studies of keratinocytes have revealed that SACCHACHITIN is able to promote its proliferation starting from the second day, and significant proliferation was observed by the fourth and fifth days. This provides further evidence that the acceleration of wound healing by the use of SACCHACHITIN is the result of proliferation of keratinocytes leading to the covering of the skin wound area. Further, the compatibility of SACCHACHITIN with keratinocytes with no sign of an immunological response (data not shown) is advantageous for its use as a wound dressing.

The elevated levels and excessive activities of MMPs in chronic wounds have recently been recognized as the principal reason for poor wound healing.[28] Activity study results of MMPs demonstrate that SACCHACHITIN and its partial monomer, *N*-acetylglucosamine, show a higher extent of inhibition of the activity of MMP-9 than of MMP-1 and MMP-8. Yanger and colleagues reported that 10- and 25-fold elevated
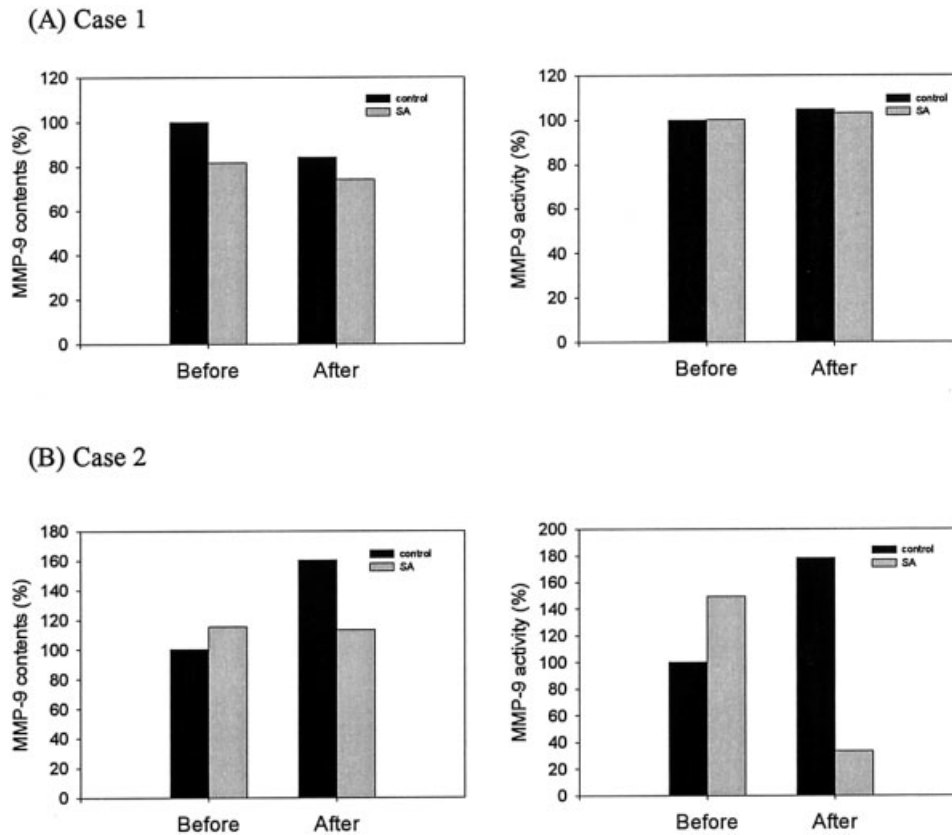
Figure 6. Quantitative analysis of MMP-9 protein levels and activities in wound tissues of two cases.

contents of MMP-1 and MMP-9, respectively, appeared in pressure ulcers, and that neutrophil-derived MMP-8 is the predominant collagenase in healing wounds and nonhealing ulcers.[26] Therefore, SACCHACHITIN might play a role as an inhibitor of the activity of MMPs (MMP-1, MMP-8, and MMP-9) in preventing degradation of the matrix structure around a wound area, leading to accelerated wound healing. However, a standard sample of chitin from crab shells showed no similar effectiveness as that by SACCHACHITIN in the inhibition of the activity of MMP-9. The component of β-glucan in SACCHACHITIN was suspected of being responsible for this difference in inhibition.

MMPs represent a family of matrix-degrading proteinases with structural similarities. MMPs share common features, including a proenzyme domain (I), a catalytic domain (II), and a C-terminal domain (III), which are thought to define substrate specificity. Catalytic Zn interacts with a conserved cysteine (C) in domain I to maintain the proenzyme in an inactive conformation. Coordination of a zinc ion at the active site is required for catalysis,[27] and activity is specifically inhibited by tissue inhibitors of matrix metalloproteinases (TIMPs).[28] Binding studies of MMPs reveal that SACCHCHITIN is able to complex with the three MMPs to significant but different extents. There-

fore, the influence of SACCHACHITIN on the activity of MMPs occurs through its binding effect by masking the catalytic domain of MMPs

SACCHACHITIN was demonstrated to promote wound healing in both animal and cell proliferation studies. Two patients with impaired wound healing for more than 7 months were recruited for treatment with SACCHACHITIN according to protocols approved by our University Institutional Review Board. None of all chronic wounds showed any adverse reactions to treatment with SACCHACHITIN, and the healing of those wounds was promoted when SACCHACHITIN was used as a wound dressing. Even though there was a limited number of samples, the content of MMP-9 in those wound tissues tended to gradually decrease after treatment with SACCHACHITIN. This is contrary to the results that elevated levels and activities of MMPs were found in pressure ulcers.[26] One might conclude that the enhanced healing of chronic wounds by SACCHACHITIN is due to suppression on the continued increases in the levels and activities of MMPs in chronic wounds.

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