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Short communication

Effects of molecular weight and deacetylation degree of chitin/chitosan on wound healing

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Abstract

We studied the effects of chitin/chitosan on wound healing with reference to chemical properties using a linear incisional wound model in rats. Wound break strength of the chitosan group (D-glucosamine (GlcN), chito-oligosaccharide (COS), chitosan) was higher than the chitin group (*N*-acetyl-D-glucosamine (GlcNAc), chiti-oligosaccharide (NACOS), chitin). Collagenase activity was also higher in the chitosan group than the chitin group. There was no significant change between the concentration of the sample and the break strength and collagenase activity in all samples. In histological findings, collagen fibers run perpendicular against the incisional line in the oligosaccharide group (NACOS, COS), and many activated fibroblasts were observed around the wound in the chitosan group. As for the deacetylation degree, the higher the deacetylation degree becomes, the more the stronger the break strength becomes. Also, activated fibroblasts appeared more in the higher deacetylation degree.

Keywords: Chitin/chitosan; Collagenase; Deacetylation degree; Molecular weight; Rat; Wound healing

1. Introduction

Chitin and chitosan have many useful and advantageous biological properties in the application as a wound dressing, namely biocompatibility, biodegradability, hemostatic activity, anti-infectional activity, and a property to accelerate wound healing. We studied the effects of chitin and chitosan on wound healing, and found that these materials induced the activation of a complement system (Minami, Suzuki, Okamoto, Fujinaga, & Shigemasa, 1998;Suzuki et al., 1997), polymorphonuclear cells (Minami et al., 1997;Usami et al., 1994a;Usami et al., 1994b;Usami, Minami, Okamoto, Matsuhashi, & Shigemasa, 1997;Usami, Okamoto, Takayama, Shigemasa, & Minami, 1998), fibroblasts and vascular endothelial cells (Okamoto et al., 2002). When chitin and chitosan are applied in the body, these materials are biodegraded by some enzymes such as chitinase and chitosanase, and subsequently become to their oligomers and monomers. Our previous results indicated that not only chitin and chitosan but also their oligomers and monomers influenced fibroblasts and endothelial cells migration. This suggests that their oligomers and monomers influence wound healing *in vivo*. However, the relationship between chemical properties of chitin/chitosan and wound healing is still unclear. In the present study, we prepared chitin and chitosan that have a different molecular weight and degree of deacetylation, and we examined the effects of them on wound healing using a linear incisional wound model in rats. We also measured break strength of the wound and collagenase activity in the tissue as an indicator of wound healing.

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2. Experimental

2.1. Reagents

Chitin and chitosan were supplied by Sunfive Co., Ltd. (Japan). Chitin (MW, 300 kD) and chitosan (MW, 80 kD) of a 3.5 μm mean particle size were sterilized using ethylene oxide gas, and suspended in phosphate-buffered solution (PBS, pH 7.2) at a concentration of 10 mg/ml. The chitin and chitosan had degrees of deacetylation of <10% and >80%, respectively. Monomers (*N*-acetyl-D-glucosamine (GlcNAc), D-glucosamine (GlcN)) and oligomers of chitin and chitosan (chiti-oligosacharide (NACOS), chito-oligosaccharide (COS)) were supplied by Yaizu Suisankagaku Industry (Japan). The NACOS and COS contained a mixture of GlcNAc1–GlcNAc5 and GlcN1–GlcN6, respectively. Each oligomers and monomers were dissolved in PBS at a concentration of 10 mg/ml and then filter sterilized through 0.45-μm filters. Each sample was adjusted to 0.1–10 mg/ml with PBS. Four different deacetylated chitins (DAC) (14%, 33%, 63%, and 96%) with the same molecular size (50 kD) were supplied by Sunfive, and they consisted of fine powder with a 6–8 μm mean particle size and were sterilized and adjusted in a same manner as chitin and chitosan. The powder was completely deacetylated (DAC 100) with the method described by Mima, Miya, Iwamoto, and Yoshikawa (1983). Homogeneous partial acetylation was performed by the method described by Hirano, Ohe, and Ono (1976). Furthemore, molecular weight was determined by the viscosity method (Tokura & Nishi, 1995) and the degree of deacetylation was evaluated using the IR method (Shigemasa, Matsuura, Sashiwa, & Saimoto, 1996) and/or the colloidal titration methods (Kina, Tamura, & Ishibashi, 1974).

2.2. Experimental design

Seventy-two Wistar female rats (300 ± 20 g) were used in this study. After back region of the animal was clipped and disinfected with iodine, 2 full-thickness incisional wounds (4 cm in length) were created with a scalpel under general anesthesia. After hemostasis was achieved, 100 μl of each sample was lavaged in the wounds and the wounds were closed with interrupted 4-0 stainless steel sutures. Seven days after the surgery, the animals were euthanized and biopsy samples from the wounded site were collected. In break strength analysis, a strip of skin (1 cm in width and 2 cm in length) was taken from the center site of the wounds in each sample. The maximum strength until breaking the incisional line was measured using a load gauge (Measurement Innovator, Aikoh Engineering, Japan) and tensiometer (Model 1356, Aikoh Engineering, Japan). The data obtained was divided by a transverse area of 5 mm of the incisional line in each sample and was expressed as a Newton per unit area (N/mm^2). Using the remaining specimen, collagenase activity in the tissue was assayed with a type I collagenase measurement kit (Yagai, Co. Ltd., Japan) and histological observation was performed. Lastly, collagenase activity was expressed as a unit per μg of protein in the tissue.

2.3. Statistical analysis

The Fisher's exact test was used to assess the significance of each group. The Student's *t*-test was used for analysis when two groups were evaluated. Moreover, a *p*-value of less than 0.05 was considered significant.

3. Results and discussion

3.1. Effect of molecular weight on wound break strength and collagenase activity

The effect of molecular weight on wound break strength is shown in Fig. 1. The wound break strengths in all samples were increased significantly compared to that of the control (saline). In both chitin (GlcNAc, NACOS, chitin) and chitosan (GlcN, COS, chitosan) groups, oligomers (NACOS, COS) were most effective. When the chitin and chitosan groups were compared, the chitosan group was higher than the chitin group in each molecular size.

The effect of molecular weight on collagenase activity is shown in Fig. 2. Collagenase activities in all samples were increased significantly compared to that of the control (saline). In the chitosan group, monomer (GlcN) was the most effective, while there was no difference in the chitin group. Collagenase activity was also higher in the chitosan group than the chitin group. There was no significant change between the concentration of the sample and wound break strength and collagenase activity as well.

In histological findings, more activated fibroblasts were observed around the wound in the chitosan group than the chitin group. Collagen fibers tended to run perpendicular against the incisional line with oligomers (NACOS, COS) (Fig. 3), while those in the control and monomers (GlcNAc, GlcN) run parallel to the incisional line (Fig. 4).

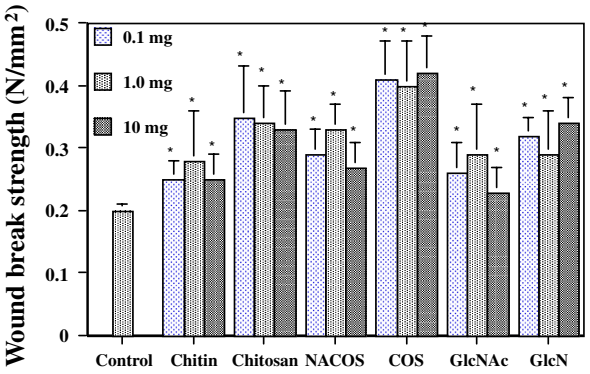

Fig. 1. Effect of molecular weight on wound break strength. Each sample was applied topically at wounding. On day 7 after wounding, wound break strength was measured using load gauge and tensiometer for maximum break strength. Results are expressed as mean ± SD ($n = 3$).

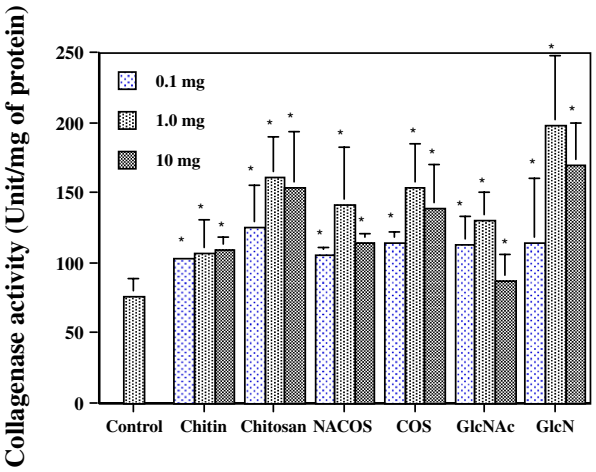

Fig. 2. Effect of molecular weight on collagenase activity. Each sample was applied topically at wounding. On day 7 after wounding, each sample was taken and assayed by type I collagenase measurement kit. Results are expressed as mean ± SD ($n = 3$).

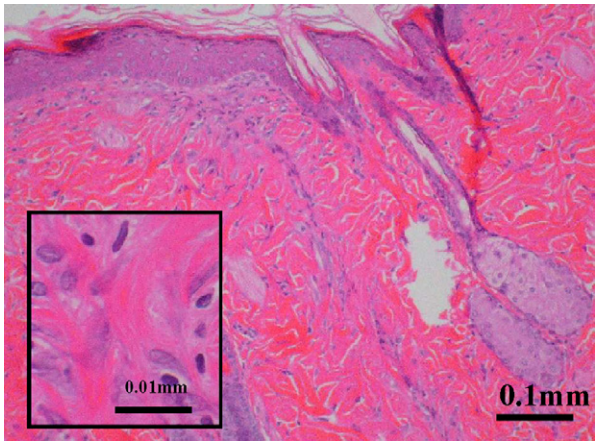

Fig. 3. Histological findings in the COS group at concentration of 1 mg/ml on day 7 after wounding. More activated fibroblasts were observed around the wound and collagen fibers tended to run perpendicular against incisional line.

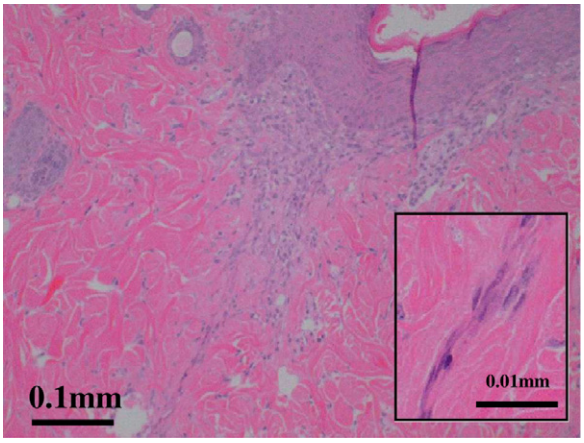

Fig. 4. Histological findings in the control group on day 7 after wounding. Fibroblasts were inactive and collagen fibers run parallel to the incisional line.

The present results indicated that not only chitin/chitosan but also their oligomers/ monomers enhanced wound healing acceleration. Previous reports indicated that chitin and chitosan increased wound healing acceleration (Minami et al., 1993;Okamoto et al., 1993;Okamoto et al., 1995) as well. However, it was unclear whether or not chitin and chitosan were directly effective because they were biodegraded by some enzymes and become oligomers and monomers in the wound. The present study proved that biodegradable substances of chitin/chitosan were effective in wound healing. In particular, oligomers were found to more effective. Histologically, it was notable that collagen fibers run perpendicular to the wound line in the oligomers groups, which suggests wound healing acceleration. Based on the present result, however, we cannot explain exactly why oligomers were more effective for wound healing. Nevertheless, one possible answer is as follows: When each sample with different molecular size was administered to the wound, the degree of biodegradability of each sample was different. Therefore, in chitin/chitosan, it takes more time to be biodegraded and absorbed in the wound due to their high molecular weight, while monomers are absorbed quickly due to low molecular weight. In addition, oligomers are also suitable in respect to absorption.

Not only chitin/chitosan but also their oligomers/monomers were found to enhance collagenase activity. Collagenase activity is related to the remodeling in the wound healing process (Clark & Denver, 1985). This enzyme is produced mainly by fibroblasts and inflammatory cells. In histological findings, more activated fibroblasts and less inflammatory cells were observed at the wound site, which suggests that fibroblasts mainly produce collagenase. This phenomenon means that chitin/chitosan and their biodegradable substances influence the remodeling phase in the wound healing process. We have many clinical studies that prove that scar formation does not occur at the wound site in the presence of chitin/chitosan (Okamoto et al., 1993; Okamoto et al., 1995). Another reseacher also indicated this same phenomenon (Ohshima, Nishino, Yonekura, Kishimoto, & Wakabayashi, 1987), however, the mechanism was still unclear. Therefore, the present result gives one explanation and posibility to this phenomenon.

In the present study, all samples at the concentration ranging from 0.1 to 10 mg/ml enhanced wound healing and collagenase activity. This phenomenum suggests that the sample at much lower concentration may be effective to wound healing. In the present study, I decided concentration range based on previous our results. In previous result, we found chitin at the weight of 0.1 mg and chitosan at the weight of 0.01 mg induced granulation tissue *in vivo* (Kojima et al., 2001). In addition, GlcN and GlcNAc at the concentration of 50 μg/ml enhanced IL-8 release from dermal fibroblasts *in vitro* (Mori et al., 1997). In the present study, we used the samples at the concentration ranging from 0.1 to 10 mg/ml and administered 0.1 ml of each sample to the wound (about 4 cm × 1 cm wound bed). Minimum weight of each sample is 0.01mg (10 μg). I thought

that this weight was reasonable, and then I designed the present experiment. As results, all concentrations are enough to accelerate wound healing. In this point, we must investigate effects of samples at lower concentration on wound healing.

3.2. Effect of deacetylation on wound break strength and collagenase activity

Table 1 shows the effects of deacetylation on wound break strength and collagenase activity. The levels of both paramers in all samples were significantly higher than that of the control. The higher the degree of deacetylation, the stronger the break strength and more collagenase activity. Histologically, more activated fibroblasts were observed in the sample with a higher degree of deacetylation (Figs. 5 and 6).

The present study proved that deacetylation influenced wound healing acceleration. As a result, we presume that amino residue is strongly related to wound healing acceleration. Histologically, more activated fibroblasts were observed. Nishimura, Ishihara, Ukei, Tokura, and Azuma (1986) reported that 70% deacetylated chitin was most effective to macrophage acitivation. Suzuki et al. (1997) indicated that a higher deacetyled chitin induced a strong complementary activity compared to lower one. The present result is consistent with the latter one. In the future, further study is necessary on the relationship between amino residue and fibroblasts activation.

Table 1
Effects of deacetylation on wound break strength and collagenase activity

Agent	Wound break strength (N/mm^2)	Collagenase activity (Unit/μg of protein)
Saline	0.21 ± 0.01[a]	76.7 ± 12.0
DAC 14[b]	0.27 ± 0.03	93.8 ± 12.0
DAC 33	0.27 ± 0.07	135.1 ± 36.6
DAC 63	0.35 ± 0.08	135.4 ± 20.4
DAC 96	0.44 ± 0.08	184.1 ± 40.9

[a] Mean ± SD ($n = 4$).
[b] DAC 14 means 14% deacetylated chitin.

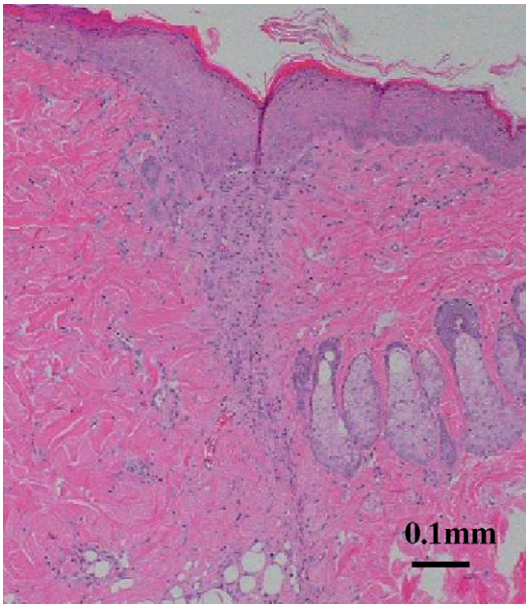

Fig. 5. Histological findings in the DAC 14 group on day 7 after wounding. Fibroblasts were inactive and collagen fibers run parallel to the incisional line.

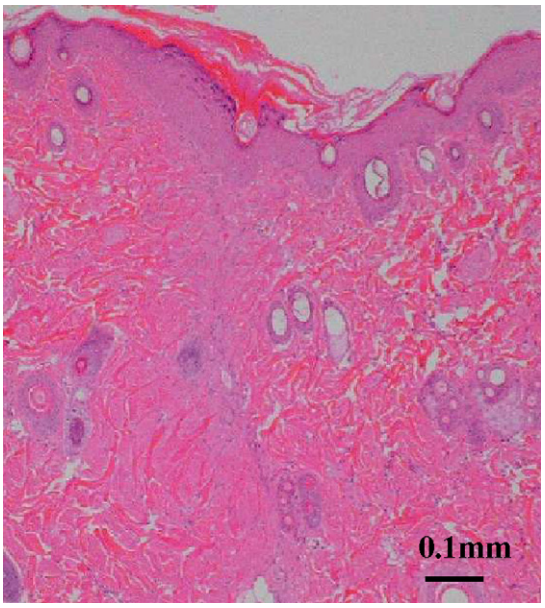

Fig. 6. Histological findings in the DAC 96 group on day 7 after wounding. More activated fibroblasts were observed in the wound.

4. Conclusion

In addition to chitin/chitosan, their oligomers/ monomers were found to enhance wound healing acceleration. Wound break strength and collagenase activity of the chitosan group (D-glucosamine (GlcN), chito-oligosaccharide (COS), chitosan) were higher than the chitin group (*N*-acetyl-D-glucosamine (GlcNAc), chiti-oligosaccharide (NACOS), chitin). In histological findings, collagen fibers run perpendicular against the incisional line in the oligosaccharide group (NACOS, COS) and many activated fibroblasts were observed around the wound in the chitosan group. In deacetylation, the higher the degree of deacetylation was, the stronger the break strength and more activated fibroblasts were observed.

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