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Collagen typing of granulation tissue induced by chitin and chitosan

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Abstract

Granulation tissue induced by chitin- or chitosan-coated polyester nonwoven fabric (chitin-NWF, chitosan-NWF) and uncoated NWF (control) implanted in the feline abdominal wall was evaluated by macroscopic and microscopic observation. Tissue growth around the implant was most marked with chitosan-NWF and least with chitin-NWF. The inflammatory reaction was much stronger with chitosan-NWF. In the tissue surrounding the implant, many giant cells and prominent angiogenesis were induced by chitin-NWF compared to the other NWFs. Types I, III and IV collagen were synthesized at higher levels in the chitin-NWF implants, especially type IV collagen, compared with the other implants. At 14 days after implantation, the levels of types III and IV collagen were also greater in the tissue surrounding the chitin-NWF. The pattern of synthesis of each type of collagen induced by chitosan-NWF in the surrounding tissue closely resembled that of the control.

Keywords: Chitin; Chitosan; Collagen

1. Introduction

It is well known that chitin and chitosan can improve wound healing in animals (Minami et al., 1993; Okamoto et al., 1993b). Wounds treated with these materials heal without excessive granulation tissue and without scar formation (Chandy and Sharma, 1990; Okamoto et al., 1993a). A lack of scarring has been observed after the treatment of serious skin trauma using chitosan (Minami et al., 1993), especially in cats. Histological findings suggest that these materials stimulate the migration of polymorphonuclear (PMN) and mononuclear cells and accelerate the growth of granulating tissue with abundant neovasculature (Muzzarelli et al., 1988; Minami et al., 1993). In the wound healing process, fibroblasts are one of the key factors involved in remodeling tissue defects by collagen production. It is currently accepted that fibroblasts follow inflammatory cells into sites of tissue injury and contribute to wound healing through the synthesis of structural proteins. These cells also facilitate wound contraction and reorganization of the extracellular matrix. We have previously reported that chitin induced the production of types III and IV collagen in tendon tissue (Minami et al., 1996, Okamoto et al., 1997). However, this is a special model of wound healing with poor vascularity. With regard to wound healing in subcutaneous tissue, chitin administration significantly increased the tensile strength of skin sutures within 5 days, compared with the control, without an increase in tissue hydroxyproline concentration (Yano et al., 1985). This suggests that the increase in tensile strength resulting from the chitin administration was not mediated by the stimulation of collagen synthesis. However, there is no information about variable collagen types in regenerated tissue after chitin application. There are also no reports concerning the collagen types induced by chitosan treatment. Furthermore, there is no data on collagen synthesis induced by chitin and chitosan in cats, which are frequently treated at veterinary clinics, as also are dogs.

In the present study, granulation tissue induced in cats by chitin and chitosan was evaluated by macroscopic and microscopic observation.

2. Materials and methods

2.1. Animals

Nine 1-year-old cats, with a mean weight of 2.5 kg, were used for this study. The animals were purchased from

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Hamaguchi Laboratory Co. Ltd (Japan). All cats were healthy based on clinical and hematological examinations. Serum antibody titers showed that they were free from the feline leukemia virus, the feline immunodeficiency virus and the feline infectious peritonitis virus.

2.2. Implants

Three type of implants were prepared, including a polyester nonwoven fabric (NWF: polyethylene/terephtalate, Du Pont, USA), a composite of chitin and NWF (chitin-NWF: chitipack P^R, Eisai Co., Japan) containing 1.4 mg/cm^3 of chitin, and a composite of chitosan and NWF (chitosan-NWF: Sunfive Co. Ltd, Japan) containing 0.1 mg/cm^3 of chitosan. Chitin was supplied by the Nippon Suisan Co. (Japan), and was purified from squid pen. Its characteristics were as follows: ash, maximum 1%; protein content, maximum 0.3%; molecular weight, 370,000; heavy metals such as Pb, Cd and As, maximum 10 ppm. Chitosan (Flonac C) was supplied by the Kyowa Tecnos Co. (Japan), and comprised 82% deacetylated α-chitin, purified from crab shell. The characteristics of this chitosan were as follows: Ash, maximum 1.2%; protein content, maximum 0.3%; molecular weight, 80,000; heavy metals such as Pb, Cd and As, maximum 5 ppm. The methods of manufacturing chitin- and chitosan-NWF were described in our previous report (Tanioka et al., 1993). These materials were sterilized with ethylene oxide gas prior to use.

2.3. Experimental design

The cats were randomly divided into three groups: A, B and C. The animals were anesthetized by the intravenous administration of 20 mg/kg ketamine-HCl following subcutaneous administrations of 0.05 mg/kg atropine sulfate and 2 mg/kg xylazine-HCl for premedication. Each cat was placed on its back and the ventral region was prepared for aseptic surgery. A long skin incision was performed from the xiphoid process to pubic bone and the subcutaneous tissue was bluntly dissected. Three full thickness defects (2 cm in diameter) were created in the abdominal wall with a scalpel: on both sides in the upper abdomen (first and third defects) and on one side in the lower abdomen (second defect). The implants were cut to form circles of 2 cm in diameter and the NWF, chitin-NWF and chitosan-NWF were placed in the first, second and third defects, respectively, in group A. In group B, the materials were placed in the second, third and first defects, respectively, while, in group C, they were placed in the third, first and second defects, respectively. The implants were fixed to the abdominal wall using eight interrupted sutures of 3-0 nylon (Akiyama Seisakusho, Japan). The skin and subcutaneous tissue were closed separately with interrupted wire sutures (0.15 mm in diameter, Mizuho, Japan) and a continuous 3-0 absorbable braided suture (Medifit, JMS, Japan), respectively.

2.4. Macroscopic observation

One cat in each group was killed with 88 mg/kg of pentobarbital (Nembutal, Dainippon Seiyaku, Japan) intravenously at 3, 6 and 14 days after surgery (days 3, 6 and 14, respectively). After the skin was removed, each implant was evaluated macroscopically and then harvested, together with the surrounding tissue, using a scalpel.

2.5. Histological observation

The implants were fixed in 10% phosphate-buffered formalin and then were dehydrated through an alcohol series and 100% xylene. After dehydration, samples were embedded in paraffin and 3–4 μm thick sections were cut. These sections were stained with hematoxylin-eosin. Collagen typing was performed by immunohistochemical staining (ABC method) for types I, III and IV collagen. The method was based on that of Minami et al. (1996). In brief, the sections were deparaffinized and rehydrated. Endogenous peroxidase activity was eliminated by exposure to 2% periodic acid for 10 min. To prevent nonspecific binding of anti-rabbit IgG goat serum, the sections were incubated for 10 min in 5% normal goat serum. For immunohistochemistry, the sections were first treated with rabbit anti-serum, for each type of collagen [anti-human (placenta) and bovine (skin) types I, III and IV collagen, LSL Co., Japan], diluted to 1:400 for 30 min at room temperature. In a preliminary experiment, we confirmed that these antisera showed a cross reaction with feline collagen. There was no cross reaction among types I, III, and IV collagen, however. Sections were then treated with peroxidase-conjugated anti-rabbit IgG goat serum diluted to 1:100 (Miles Labo. Inc., Japan) for 30 min at room temperature, colored with a diaminobenzidine for 10 min and counterstained with hematoxylin solution.

2.6. Histomorphometric evaluation

The thickness of the implants was measured using a video micrometer (VM31, Olympus, Japan) in each hematoxylin-eosin stained section. In brief, the thickness at five random sites was measured in each section and the values were averaged.

Image analysis of collagen-stained sections was performed by the method of Minami et al. (1996), using an image processor (MV 4000, Nippon Data General Co. Ltd, Japan) and a multicolor data system (4200F, NAC Co. Ltd, Japan). Randomly selected histological images obtained with a light microscope at 100 $\times$ magnification from three fields were directly transmitted via a TV camera into the MV 4000, and were recolored to two colors using the XYGDMP and GCONVERT programs. Recoloring was performed as follows: (1) the background (unstained interstitial space and polyester fibers) intensity of each specimen was set at the same gray level for all input fields. (2) Then

the gray scale distribution of the stained area was measured. (3) The entire input area was divided into positive and negative regions and two-color histograms were analyzed by the HGRAM program. The mean frequency of each color was obtained for the three inputted areas. Finally, each color was expressed as the number of pixels per 100 000 pixels of the input field.

2.7. Statistical analysis

Statistical analysis was performed using Student's *t*-test.

3. Results

3.1. Macroscopic findings

On day three, a small amount of reddish exudate was accumulated on the chitosan-NWF implants and edema was observed in the surrounding tissues. There was no exudate or edema of the surrounding tissues in the case of the control and chitin-NWF implants. Slight adhesions were seen on both sides of the chitin-NWF and control implants, but only on the visceral side of the chitosan-NWF implants. On day six, granulation tissue was proliferating around all of the implants. In the case of chitin-NWF implants, the tissue was thin and smooth compared to the other implants. Strong adhesions on the outer side and obvious angiogenesis on the adhesion side were observed in all implants. On day 14, the chitosan-NWF implants showed prominent proliferation of granulation tissues and much stronger adhesions on the outer side. In the case of chitin-NWF implants, very smooth regeneration was observed. With the control implants, the regeneration was greater than with the chitin-NWF, but not greater than with the chitosan-NWF.

Data on implant thicknesses are shown in Table 1. With all the implants, a gradual increase in thickness occurred over time. The chitin-NWF implants were the thinnest during the experiment. A significant difference was observed between the control and chitin-NWF implants on day 6 and between the chitin-NWF and chitosan-NWF implants on day 14.

Table 1
Thickness of the implants

Implant	Day[a]		
	3	6	14
NWF	1063 ± 120[b*]	1346 ± 88[*]	2931 ± 428[*,†]
Chitin-NWF	895 ± 37[*]	975 ± 75[†]	1902 ± 659[*]
Chitosan-NWF	1117 ± 180[*]	1148 ± 316[*,†]	3869 ± 672[†]

[a]Post-implantation day.
[b]Mean ± SD (μm).
Different superscript means significant ($p < 0.05$).

Table 2
Histological findings of the implants

Day	Component	NWF	Chitin-NWF	Chitosan-NWF
3	Fibroblast	−	−	−
	Inflammatory cell	+	+ +	+ +
	Giant cell	−	−	−
	Histiocyte	−	−	−
	Angiogenesis	−	−	−
6	Fibroblast	+	+ +	+ +
	Inflammatory cell	+	+	+ +
	Giant cell	+	+ +	+
	Histiocyte	+	+	+
	Angiogenesis	+	+ +	+ +
14	Fibroblast	+ + +	+ + +	+ + +
	Inflammatory cell	+	+	+ +
	Giant cell	+ + +	+ + +	+ + +
	Histiocyte	+ + +	+ + +	+ + +
	Angiogenesis	+ +	+ + +	+ + +

(−): Not observed.
(+): Observed only at the border site of the implant.
(+ +): Slight appearance in the whole area.
(+ + +): Numerous appearances in the whole area.

3.2. Histological findings

The histological findings of the implants are summarized in Table 2. On day three, a slight amount of granulation tissue was observed around the implant margins in all specimens. At the central area of the implants, some inflammatory cells were observed. On day six, granulation tissue had invaded to the central area of the chitin- and chitosan-NWF implants, but not the control implants. At this time, giant cells were observed in all the specimens. Inflammatory cells (PMN cells) were more prominent in the chitosan-NWF implants compared with the other implants. On day 14, inflammatory cells had disappeared from the chitin-NWF and control implants, but were still diffuse in the chitosan-NWF implants. Although chitin particles had disappeared from the implants within six days, chitosan particles were observed in the chitosan-NWF implants throughout the experimental period.

The histological features of the tissue surrounding the implants are summarized in Table 3. On day three, little

Table 3
Histological findings of tissue surrounding implants

Day	Component	NWF	Chitin-NWF	Chitosan-NWF
6	Inflammatory cell	+ + +	+ +	+ + +
	Giant cell	+	+ +	+
	Histiocytes	+	+	+
	Angiogenesis	+	+ +	+
14	Inflammatory cell	+ +	+ +	+ +
	Giant cell	+	+ +	+
	Histiocyte	+	+ +	+
	Angiogenesis	+	+ +	+ +

(+): Slightly observed.
(+ +): Moderately observed.
(+ + +): Numerously observed.

granulation tissue formation was observed around the implants. On day six, there was slight to moderate granulation tissue formation, with diffuse inflammatory cells being observed in all the implants. At this time, giant cells and angiogenesis were prominent in the chitin-NWF implants compared with the other implants. On days six and 14, histiocytes accumulated at the borders of the implants. This phenomenon was more prominent with the chitin-NWF implants, compared with the other implants.

3.3. Image analysis of collagen

Image analysis of each type of collagen in the implants and the surrounding tissue gave the results shown in Figs. 1 and 2. The chitin-NWF implants had higher levels of every type of collagen, compared with the other implants, throughout the experimental period, especially type IV collagen. The chitin-NWF implants showed higher levels of types III and IV collagen in the surrounding tissue on day 14. The collagen profiles of the chitosan-NWF and control implants remained similar during the experiment.

4. Discussion

The present study revealed that granulation tissue formation was stimulated markedly in the implants by chitin and chitosan, and in the surrounding tissue by chitosan. The effect of stimulation on the surrounding tissue was more limited with the chitin-NWF implants, even compared with the control implants. From the fact that inflammatory cells and chitosan particles were always observed in the implants, it appears that a continuous inflammatory response to chitosan leads to excessive granulation tissue formation. We have some experience of excessive granulation tissue formation by the continuous application of chitosan to wounded animals (Okamoto et al., 1992). The present results were consistent with our previous observations.

Regarding the less extensive granulation tissue formation in chitin-NWF, three possibilities can be suggested as follows: (1) inhibition of collagen synthesis, (2) enhancement of collagen digestion, and (3) a decreased foreign body reaction to the implant. The first possibility is not consistent with the present results, because all the collagen types showed higher levels in the chitin-NWF implants compared with the other implants. This means that organization of NWF was most active in the chitin-NWF implants compared with the others, so the third possibility was supported by our results. We have already shown that chitin-coated NWF is organized more quickly than uncoated NWF (Okamoto et al., 1992). The second possibility is supported by the finding of more giant cells and histiocytes numbers in the chitin-NWFs than in the other implants. These cells are well known for releasing collagenase enzymes (Clark and Denver, 1985; Stetler-Stevenson, 1990). Also, the cytoplasm of many giant cells was stained by anti-collagen antibodies, so these findings would suggest that giant cells phagocytosed regenerated collagen fibers and digested the

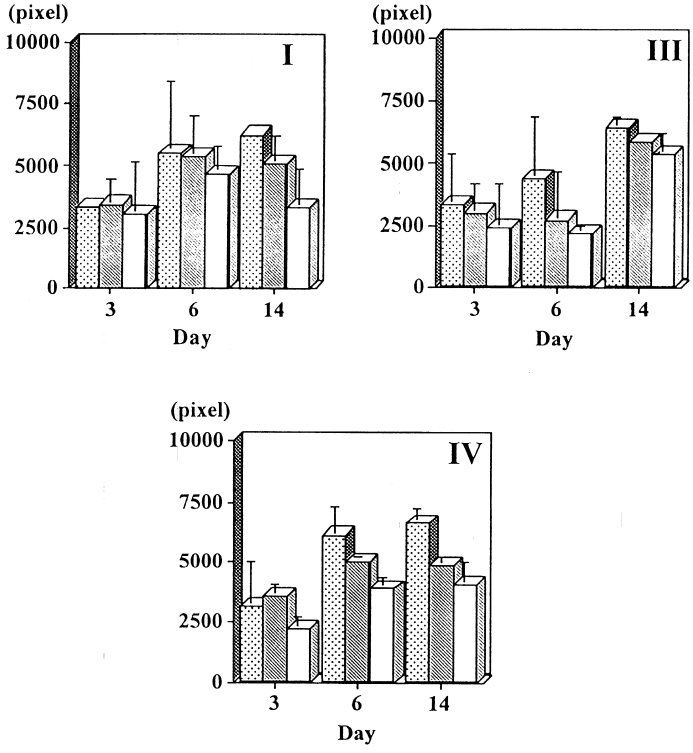

Fig. 1. Change in each type of collagen in implant.

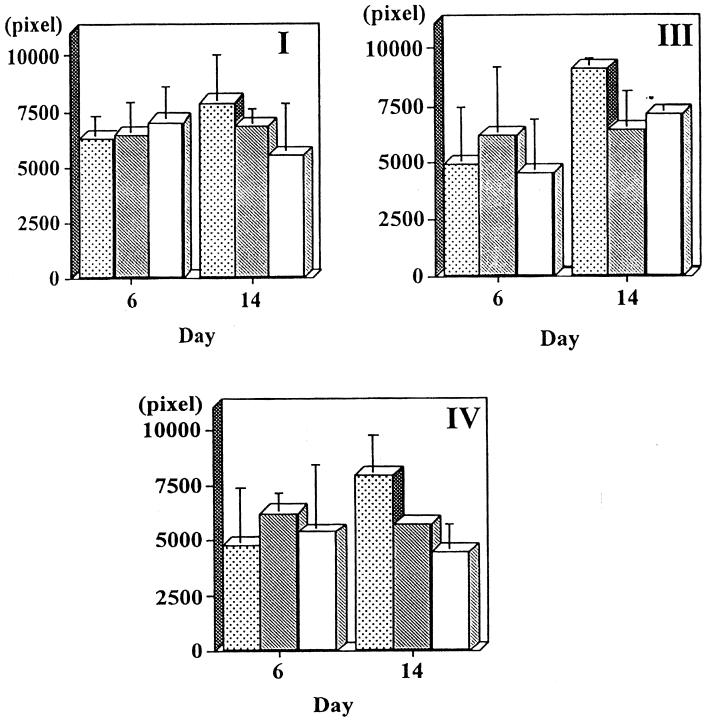

Fig. 2. Change in each type of collagen in surrounding tissue.

fibers in their cytoplasm. Similar findings have already been observed in tendon repair experiments (Minami et al., 1996; Okamoto et al., 1997). In the present study, we did not analyze collagenase levels in the tissue. In the future, it would be useful to determine the tissue collagenase concentration after chitin and chitosan implants are inserted.

The macroscopic data showed that granulation tissue formation in the surrounding tissue was greater in the presence of chitosan. However, the production of each collagen type was similar among the three implants, which suggests that the composition of collagen in the surrounding tissue was similar. In comparison with collagen synthesis in bovine tendons (Minami et al., 1996), more type I collagen was induced in the present experiment. This suggests that collagen maturation occurs more quickly in a tissue with a good vascular supply.

Chitosan created excessive granulation tissue, especially around the implant, even if the amount of chitosan in the implant was only one tenth that of chitin. This was probably because of the continuous stimulation by chitosan, resulting from its poor biodegradability, compared with chitin. Investigation of a more suitable amount of chitosan to use in implants is required.

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