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Chitin induces type IV collagen and elastic fiber in implanted non-woven fabric of polyester

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The non-woven fabric of polyester (control) and the composite material of the non-woven fabric of polyester and chitin (Chitipack P) were implanted to bovine flexor tendon. After 3 weeks implantation, type IV collagen and elastic fibers were significantly increased and type I collagen was decreased in Chitipack P in comparison with control. The breaking strength was about twice as high in Chitipack P than in control. The polykaryocytes in the control were more difficult to digest for the collagens. Angiogenesis in the implanted non-woven fabric and in the neighboring resected tendons was much stronger in Chitipack P. Chitin induced type IV collagen and elastic fibers in the prostheses. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Polymeric *N*-acetyl-D-glucosamine, chitin, has been well known as a wound healing accelerator in humans (Kifune, 1992) and various animals (Minami *et al.*, 1992; Okamoto *et al.*, 1993b). In Japan, 5 types of chitin remedies have already been marketed under the trade names Beschitin W, Beschitin F, Beschitin cotton type (Unitika, Japan), Chitipack S and Chitipack P (Eisai, Japan). These remedies are suitable for wound treatment; not only wound healing acceleration but also minimum scar formation (Okamoto *et al.*, 1993b). We have investigated wound healing effects including cytokine production (Tanigawa *et al.*, 1992) and activation of inflammatory cells in animals (Usami *et al.*, 1994a, b). We have also clarified that subcutaneous implanted chitin induced high concentrations of prostaglandin E2 (Minami *et al.*, 1995) which was one of the biological mediators of angiogenesis (Harada *et al.*, 1994). We have reported the regeneration of a vascular granulating tissue in a wounded site experimentally and clinically (Okamoto *et al.*, 1993a, b). However, it is not yet clear how chitin controls the scar formation. In order to clarify this problem, an experiment was performed to discover what kind of scar chitin will induce in an artificial material.

MATERIALS

Chitipack P (Eisai, Japan) which is a composite of chitin and non-woven fabric of polyester and contains 1.4 mg/cm^3 of chitin. The non-woven fabric of polyester (Eisai, Japan) of Chitipack P which contains no chitin was also prepared for control. Injections for anesthesia were 0.05% atropine sulfate solution (Atropine sulfate injection, Tanabe, Japan), 2% xylazine-HCl solution (Celactal, Bayer, Japan), Isoflurane (Forane, Dinabot, Japan), oxygen gas and nitrous oxide gas. Suture materials were monofilament nylon (Suprylon, USP 2, USP 1-0, Pfrimmer, Germany) and cat gut (Nesco suture, USP 1-0, Nihon Shoji, Japan).

EXPERIMENTAL

A tendon prosthesis model was made for three healthy calves, 6 months old, each weighing approximately 200 kg. Normal superficial and deep digital flexor tendons were resected (3 cm gap). The general anesthesia was performed by the inhalation anesthesia via intratracheal tube (GOI) after intramuscular induction anesthesia with xylazine (0.2 mg/kg) and atropine sulfate (0.1 mg/kg, s.c.) pre-medication. Each calf was placed in

right recumbency and the metacarpal region of each forelimb was prepared for aseptic surgery. A 6 cm straight skin incision was made over the midline of palmer aspect. The super and deep flexor digital tendons were exposed, the paratendon was incised and tendons of 3 cm were resected. Limbs were cast for 3 weeks. After euthanasia, the deep flexor tendons were recovered from both limbs and were dissected into two parts at the center line by a surgical scalpel. These parts were used for a tensile strength and a histological examination.

The tendon prosthesis

Two tendon prostheses were prepared. One is Chitipack P for a right forelimb and the other is a non-woven fabric of polyester (control) for a left forelimb. The deep flexor digital tendon ends and the prosthesis ends were stabilized with a single locking loop suture of No. 2 size monofilament nylon suture. The paratendon was closed with 1-0 size nylon. The subcutaneous tissue and skin were apposed with 1-0 size cat-gut and No. 2 size monofilament, respectively.

Mechanical testing

The tensile strength was measured by the hydraulic test machine (Autograph 5000A, Shimazu, Japan). Tensile loads were applied at 30 mm/min. The load was plotted against ram displacement with an x–y plotter. Breaking load (newton) was represented by the maximal load.

Histological examination

Sections were cut longitudinally, embedded in paraffin, sectioned at 5 mm, stained by the hematoxylin and eosin method and by the elastika-van Gieson method. Collagen typing was performed by the immunohistochemical stain (ABC method) for the types I, III and IV. The method of the collagen typing is described briefly as follows. The sections were deparaffinized and hydrated. The endogenous peroxidase activity was eliminated by exposure to 2% periodic acid for 10 min. To prevent nonspecific bindings of anti-rabbit IgG goat serum, the sections were incubated for 10 min in 5% normal goat serum. For immunohistochemical determination, the sections were treated first with anti serum to each type of collagens (Anti-human (placenta) and bovine (skin) Type I, III and IV collagens, LSL Co., Japan) produced in a rabbit and diluted to 1:400 for 30 min at room temparature. These anti-serums showed cross reactions in each collagen type among human, bovine, pig, guinea pig, rat, and mouse. There was no cross reaction among I, III and IV. They were then treated with peroxidase conjugated anti-rabit IgG goat serum diluted to 1:100 (Miles Labo., Inc., Japan) for 30 min at room temperature, colored by a diaminobenzidine reaction for 10 min and counter stained with Hematoxylin.

Polykaryocytes were microscopically divided into three grades as follows: −: no immunochemical positive granules in the cytoplasm, +: granules existed in the cytoplasm but not fully in whole cytoplasm, + +: the whole cytoplasm was stained with granules (Fig. 2). Two hundred polykaryocytes in implanted materials were counted under a 400 powered magnification of light microscope and divided into each type.

Histomorphometric evaluation

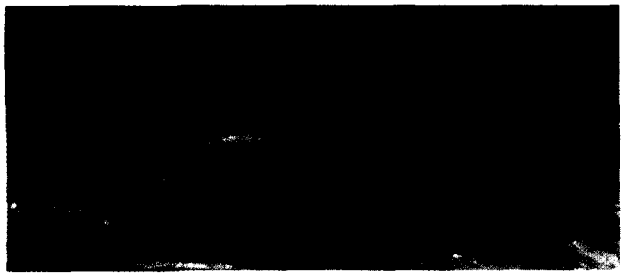
Imaging analysis of collagen typing stained sections was performed with the image processor MV 4000 (Nippon Data General Co. Ltd) and multi color data system 4200F (NAC Co. Ltd). The analysis procedures are described briefly as follows. Randomly selected histological images under a light microscope of 100 powered magnification in three fields were directly input through a TV camera into MV 4000, and recolored into 2 colors using XYGDMP and GCONVERT programs. The recoloring was performed as follows: (1) A back ground (ABC-negative area:Interstitial space and polyester fiber) intensity of each specimen was set in the same distribution of gray revel in all imputed fields. (2) The gray revel distribution in the ABC-positive area was measured. (3) The whole imputed area was divided into the ABC-positive level and the other level. Histograms of two colors were analyzed by HGRAM program. Mean frequency of each color was obtained from three imputed areas. Finally, each frequency was expressed in number of pixels in 100 000 pixels of imputed field.

The histogram analyses of elastika-van Gieson stained sections especially in elastic fibers and collagen fibers were performed by the same method as described as above.

RESULTS

Macroscopic findings of the operative digital skin after 3 weeks fixation with cast: Surgical wound was completely healed in both legs (Fig. 1). There were no obvious differences in invasion of connective tissue into the implanted materials between them. Maximum tensile strengths (newton) of flexor digital tendons were summerized in Table 1. The mean tensile strength of Chitipack P was approximately twice that of the control.

Histologically, in the hematoxylin and eosin stained specimens, there were obvious differences in the number of invaded polykaryocytes to non-woven fabric between them. The number of polykaryocytes invading Chitipack P was more than that for control. The number of each type of polykaryocyte was shown in Table 2. Polykaryocytes with positive stained cytoplasm of each collagen typing, were more in control than in Chitipack P. In the elastica-van Gieson stained sections, the elastic



Control

Chitipack-P 3 weeks

Fig. 1. The longitudinal sections of the recovered tendons. Long arrow: Chitipack P, Short arrow: Non-woven fabric of polyester (control). Both sections are after 3 weeks fixation.

Table 1. Maximum tensile strength (newtons) of flexor digital tendons

Calf No.	Chitipack P	Control
1	381	173
2	320	198
3	358	136
mean	353	169
S.D.	37	31

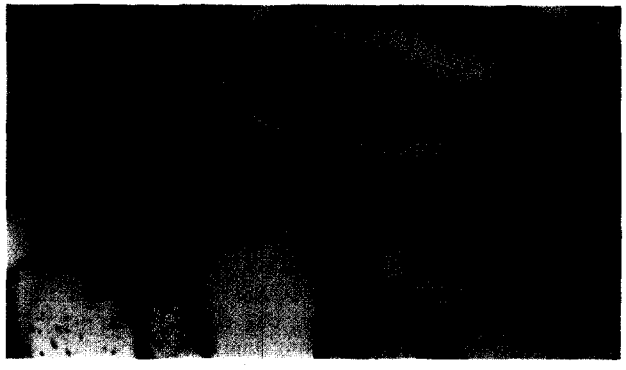


Fig. 2. Differentiation of polykaryocytes in the collagen typing immunohistochemical stained section. Long arrow: +++, three plus positive cell; Middle arrow: ++, two plus positive cell; Short arrow; −, negative cell.

fibers were more abundant in Chitipack P than in the control, especially in the connecting area between tendon and prosthesis (Figs 3 and 4).

The results of the imaging analysis of each collagen typing were shown in Table 3. The frequency of collagen type I was significantly decreased in Chitipack P than in control. In contrast, type IV collagen was significantly more increased in Chitipack P than in control. On the other hand, there was no difference in the amount of type III collagen between them. The results of the imaging analysis of elastic fiber and collagen fiber are shown in Table 4. The frequency of elastic fibers was significantly more increased in Chitipack P than in control. In contrast, the frequency of collagen fibers was more significantly decreased in Chitipack P than in control.

DISCUSSION

Tendon is one of poor vascular cicartricial tissues in a body, and so shows a late wound healing process in comparison with other organs. Tendon tissue consists of mature collagen and poor connective tissues, so it is very suitable tissue for observation of angiogenesis and collagenation against an implanted material. We have already investigated the fate of this material in the canine subcutaneous tissue and observed a complete organization in Chitipack P (Okamoto *et al.*, 1993) within 18 days implantation. In this experiment, Chitipack P and control were well organized between dissected tendons after 3 weeks implantation.

Histologically, an invasion of connective tissues had occurred from dissected proximal and distal ends of the tendon and surrounded paratendon. In Chitipack P, increasing type IV collagen corresponded well with angiogenesis, because major locality of type IV collagen in the tissue was the basement membrane of vessels (Almeida *et al.*, 1992; Schlingemann *et al.*, 1991). Kishimoto & Tamaki (1987) have also reported that fine bundle collagen reconstructed in the chitin administered wound, whereas, in the control un-chitin treated wound thick mature collagen reconstructed. Furthermore, they proposed that matured type I collagen might not be synthesized in the chitin treated wound and this is one of the reasons why the scar tissue did not form. This speculation was well supported by the results in this experiment. On the other hand, we have been pointed out that giant cells appeared in the chitin and chitosan administered wound (Minami *et al.*, 1993; Okamoto *et al.*, 1993), but their roles in wound healing had not been discussed. In the immunohistochemical stain section for collagen, the cytoplasm of it was clearly stained positively against type I, III and IV. This means the digestion of collagen by these cells. Many more positive cells appeared in control than in Chitipack P. From the results of containing elastic fibers and collagen fibers in elastica van Gieson stained sections, collagen fibers were much less in Chitipack P than in control. This will be the reason why many positive giant cells appeared in the control. The elastic fiber is the second major fibrous

Table 2. Number of polykaryocytes in the collagen typing stained sections

	Type I						Type IV					
	Chitipack P			Control			Chitipack P			Control		
Cow No.	+ +	+	–	+ +	+		+ +	+	–	+ +	+	–
1	10	84	6	83	17	0	21	58	21	60	39	1
2	11	82	7	77	24	0	13	56	31	58	40	2
3	17	60	23	81	19	0	24	70	6	69	30	1
mean	13	75	12	80	20	0	19	61	19	62	36	1
S.D.	3.8	13.3	9.5	3.1	3.6		5.7	7.6	12.6	5.8	5.5	0.6

Table 3. Imaging analysis of collagen typing stained sections between NWF(control) and C-NWF(chitin)

Collagen typing	I		III		IV	
calf No.	control	chitin	control	chitin	control	chitin
1	4435*	3845	8638	9012	8591	9208
2	4164	3556	9172	9227	8706	9354
3	4181	3440	9176	9292	8972	9455
mean	4260	3614	8995	9177	8756	9340
S.D.	124	170	253	120	160	101
welch's t	5.32		1.12		5.34	
p	0.006		0.34		0.013	

*number of pixels in 100 thousand pixels.
S.D. means standard deviation. The statistical analysis was performed between mean pixels of control and of chitin by welch's t test. Values are significantly different when P is under 0.05.

Table 4. Imaging analysis of elastic fibers and collagen fibers in the elastica van Gieson stained sections between NWF(control) and C-NWF(chitin)

fibers	elastic fibers		collagen fibers	
calf No.	control	chitin	control	chitin
1	2233*	4876	7767	5124
2	1898	5357	8102	4643
3	1600	5441	8400	4559
mean	1910	5225	8090	4775
S.D.	317	305	317	305
welch's t	13.05		13.05	
p	0.0002		0.0002	

*number of pixels in 100 thousand pixels.
S.D. means standard deviation. The statistical analysis was performed between mean pixels of control and of chitin by welch's t test. Values are significantly different when P is under 0.05.

Fig. 3. After 3 weeks implantation of Chitipack P, numerous elastic fibers generated among non-woven fabric of polyester (elastica van-Gieson stain, × 187).

Fig. 4. After 3 weeks implantation of control non-woven fabric of polyester, numerous collagen fibers generated among non-woven fabric of polyester, but elastic fibers were few (elastica van-Gieson stain, × 187).

component of connective tissue and contains two unique proteins which are protein elastin and the microfibrillar material (Peacock, 1984). The role of elastic fiber in tissues was well documented in many previous papers. Uitto (1986) pointed out that the number of elastic fibers decreases with age, and aged skin becomes loose and baggy because the collagen network becomes oriented by gravity or by muscular pull, and there is little or no restoring force. Thus elastic fibers or elastin imparts elasticity to tissue. In the hypertrophic scar tissue, incomplete elastic fiber network was responsible for the reduced capacity to return strain energy (Dunn *et al.*, 1985). On the other hand, the collagen network of tendons is normally held in a folded conformational state by elastin and the elongation of tendons is observed even in the absence of

loading after complete removal of elastin (Oakes & Bialkower, 1977). In this experiment, the tensile strength increased significantly more in Chitipack P than in control. This is responsible for the amount of elastic fibers in NWF. It was reported that an administration of transforming growth factor-beta 1 (TGF-beta 1) to wounds enhanced elastin mRNA expression (Quaglino *et al.*, 1990) and stimulated wound healing (Quaglino *et al.*, 1991). It was apparent that chitin induced elastic fibers in the implanted NWF. However, a mechanism for this has not still been clarified. Further investigations must be performed.

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