classified as a Type-IV synostosis (anterior dislocation of the radial head) according to the system of Cleary and Omer[5]. For all patients, the position of the forearm synostosis was near neutral forearm rotation. After excision of the annular-ligament-like structure and detachment of the anterior capsule, the contracture resolved and all patients were subsequently able to extend the elbow fully.

In the classification of congenital disorders of the upper limb, congenital proximal radioulnar synostosis has been grouped with disorders that are associated with the failure of differentiation of specific anatomical parts[11]. In our two patients, it appeared that the annular-ligament-like structure overlapped and became fixed to the anterior capsule. Excision of the structure released the contracture. When an acute elbow flexion contracture develops in a patient who has a Type-IV congenital proximal radioulnar synostosis, excision of the hypoplastic annular-ligament-like structure should be strongly considered. ■

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Feasibility of chitosan-based hyaluronic acid hybrid biomaterial for a novel scaffold in cartilage tissue engineering ☆

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Abstract

In this study, we hypothesized that hyaluronic acid could provide superior biological effects on the chondrocytes in a three-dimensional culture system. To test this hypothesis, we investigated the in vitro behavior of rabbit chondrocytes on a novel chitosan-based hyaluronic acid hybrid polymer fiber. The goal of the current study was to show the superiority of this novel fiber as a scaffold biomaterial for cartilage tissue engineering. Chitosan polymer fibers (chitosan group) and chitosan-based hyaluronic acid hybrid polymer fibers (HA 0.04% and HA 0.07% groups, chitosan coated with hyaluronic acid 0.04% and 0.07%, respectively) were originally developed by the wetspinning method. Articular chondrocytes were isolated from Japanese white rabbits and cultured in the sheets consisting of each polymer fiber. The effects of each polymer fiber on cell adhesivity, proliferation, morphological changes, and synthesis of the extracellular matrix were analyzed by quantitative a cell attachment test, DNA quantification, light and scanning electron microscopy, semi-quantitative RT-PCR, and immunohistochemical analysis. Cell adhesivity, proliferation and the synthesis of aggrecan were significantly higher in the hybrid fiber (HA 0.04% and 0.07%) groups than in the chitosan group. On the cultured hybrid polymer materials, scanning electron microscopic observation showed that chondrocytes proliferated while maintaining their morphological phenotype and with a rich extracellular matrix synthesis around the cells. Immunohistochemical staining with an anti-type II collagen antibody demonstrated rich production of the type II collagen in the pericellular matrix from the chondrocytes. The chitosan-based hyaluronic acid hybrid polymer fibers show great potential as a desirable biomaterial for cartilaginous tissue scaffolds.

Keywords: Chitin/chitosan; Chondrocyte; Hyaluronic acid/hyaluronan; Cell culture; Cell adhesion

1. Introduction

In Living organisms, the authentic substrate for most cells is the extracellular matrix (ECM). The ECM adheres to cells via integrins, which are membrane-spanning heterodimeric receptors [1]. Through the cell–matrix adhesions, the ECM transduces physiological signals regulating cell growth, cell proliferation, cell differentiation, and matrix remodeling to the cells [1]. Therefore, the ECM plays an important role in living tissue development and regeneration.

In a tissue engineering technique, tissue regeneration is achieved by culturing isolated cells on biocompatible and biodegradable materials as scafollds onto which cells are seeded. A large number of studies have shown the importance of selecting the appropriate biomaterials as scaffolds for the cell adhesion and supporting the proliferation [2–10]. For the reason given above, the ideal scaffold material should be one which closely mimics the natural environment in the tissue-specific ECM [9].

Once damaged, the articular cartilage consisting of hyaline cartilage tissue has little capacity for spontaneous

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healing. Although the limited potential for self-repair of the articular cartilage necessitates operations to treat injured cartilage, no current procedures for cartilage repair have successfully regenerated long-lasting hyaline cartilage tissue to replace a cartilaginous lesion [11,12]. To solve this limitation, tissue engineering techniques by culturing isolated chondrocytes on a variety of scaffold materials, including naturally occurring and synthetic, have been developed [4–10]. However, there have been no ideal materials for cartilage tissue engineering.

One of the considerable characteristics in the cartilage tissue is that a small number of chondrocytes are embedded in the rich ECM. Therefore, cell–matrix interactions play a crucial role in the development and regeneration of the cartilage tissue. To successfully achieve cartilage tissue regeneration, a cell-carrier substance which closely mimics the natural environment in the cartilage-specific ECM must be developed. In the current study, hyaluronic acid, which is a main component of the proteoglycans (PGAs) in the cartilage, was applied to chitosan as a fundamental biomaterial.

Recently, several studies have demonstrated that cellular functions differ in two-dimensional and three-dimensional (3D) systems [13,14]. In cartilage tissue engineering, a closer approximation to in vivo environments should be attained by culturing cells in 3D materials. Additionally, the articular cartilage must be considered for its mobility as an excessively stressed tissue. To structurally mimic the environments of the cartilage tissue, the fundamental structure of a scaffold should be a 3D system with adequate mechanical strength. In the current study, the authors have structurally developed a novel polymer fiber—chitosan-based hyaluronic acid hybrid fiber—as a biomaterial to easily fabricate 3D scaffolds.

In this study, we hypothesized that hyaluronic acid could provide superior biological effects on the chondrocytes in a 3D culture system. To test this hypothesis, we investigated the in vitro behavior of rabbit chondrocytes on a novel chitosan-based hyaluronic acid hybrid polymer fiber. The objectives of the current study were to evaluate the chondrocyte adhesion, proliferation, and the synthesis of the ECMs in the chitosan-based hyaluronic acid hybrid polymer fiber and to show the superiority of this novel fiber as a scaffold biomaterial for cartilage tissue engineering.

2. Materials and methods

2.1. Polymer fibers

Polymer fibers were developed by the wet spinning method as described by Tamura et al. [15] with the following modification. Fig. 1 shows the process of

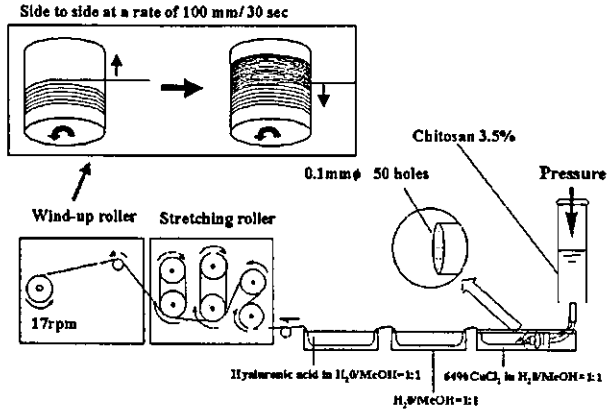


Fig. 1. The original roller system.

developing the fibers using an original apparatus [15]. Chitosan is a commercial material purchased from Kimitu Chemical Co. Inc., (Tokyo, Japan). Hyaluronic acid produced by lactic acid bacteria, with a viscosity average molecular weight of 2,400,000, was gifted from DENKI KAGAKU KOUGYO Co. Ltd. (Tokyo, Japan). The degree of deacetylation of the chitosan was 81%, and viscosity average molecular weight was 600,000. To prepare the polymer fiber 7 g of chitosan powder was dissolved in 200 ml of 2% aqueous acetic acid solution to give 3.5% of polymer concentration. Dope of chitosan was spun into a calcium coagulant bath (64% $CaCl_2$ dissolved in 50% aqueous methanol solution) through a stainless steel spinnlet (0.1 mm diameter, 50 holes) at a winding speed of 4.4 m/min at room temperature. Then, 50% aqueous methanol solution was used as a second coagulation bath and 0.04 or 0.07% hyaluronic acid dissolved in 50% aqueous methanol solution was a third coagulation bath. Using an original roller system (Okada Co. Inc., Sapporo, Japan), the resulting fibers were stretched and treated with 0.8% sodium hydroxide (NaOH) dissolved in 90% aqueous methanol solution to neutralize the acidity of the fibers. The fibers wound in the roller were washed with methanol and dried at room temperature. The diameter of each fiber was 0.03 mm.In the current study, chitosan polymer fiber (chitosan group) and chitosan-based hyaluronic acid hybrid polymer fiber (chitosan coated with hyaluronic acid 0.04%, HA0.04% group; chitosan coated hyaluronic acid 0.07%, HA0.07% group) were originally developed. For further investigations of the chondrocyte culture system, we automatically made a fiber sheet using the original apparatus (Fig. 1). Coagulated fibers were passed through a cross feeding guide and wound onto a stainless roller (120 mm diameter and 120 mm wide) at the rate of 17 rpm. The cross feeding guide set forward in the roller was moved from side to side at a rate of 100 mm/30 s. The cross-feed length and rotation count were 100 mm and 40 times,

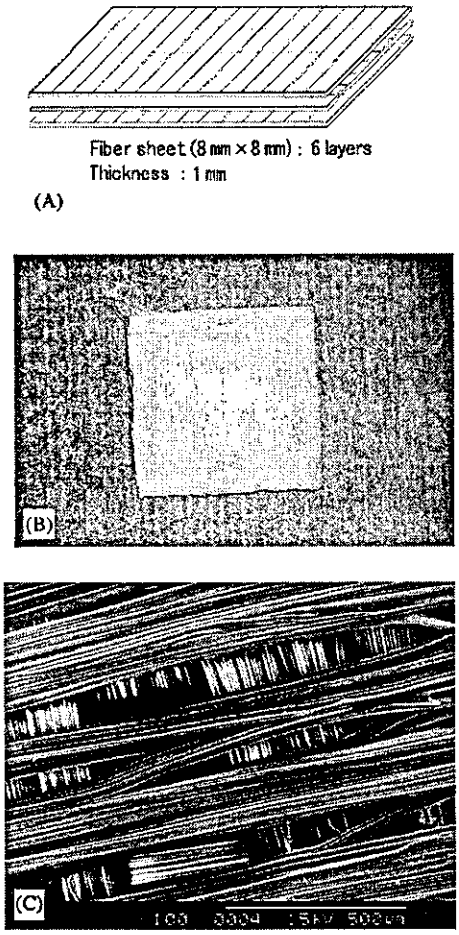


Fig. 2. (A,B) Three-dimensional (3D) scaffold material for the chondrocyte culture. Sheets consisting of each polymer fiber are stacked in a perpendicular pattern with six layers (8 × 8 mm, 1 mm thickness). (C) Scanning electron micrograph of the 3D scaffold material.

respectively. The fibers wound onto the roller were washed and dehydrated with methanol, and then dried at room temperature. The dry filaments were cut from the roller and a sheet fiber of filaments were piled 40 high (380 mm length, 100 mm wide and 0.25 mm thickness). The sheets consisting of each polymer fiber were cut into small sheets and stacked in a perpendicular pattern with six layers (8 × 8 mm, 1 mm thickness) (Fig. 2A,B,C). Each sheet was peripherally adhered with 2% chitosan gel and dried at 40°C for over night. During this process, we constantly obtained the same 3D fabrications. These 3D materials were sterilized in autoclave at 135°C for 20 minutes for the chondrocyte culture.

2.2. Measurement of material properties

Material properties of five samples in each fiber group were measured according to the Japanese Industrial Standards L1015. Tensile tests for each fiber group were performed at a crosshead speed of 20 mm/min using a material testing machine (P/N346-51299-02, SHIMADZU, Kyoto, Japan). The cross-sectional area was determined using a microscope (BX50, OLYMPUS, Tokyo, Japan) and a video dimension analyzer (VM-30, OLYMPUS, Tokyo, Japan).

2.3. Chondrocyte suspension

Ten week old Japanese white rabbits with a mean body weight of 2.0 kg (Hokudo, Sapporo, Japan) were used in this study. Articular cartilage slices, gathered from the knee, the hip, and the shoulder joints of each animal, were detached from the adherent connective tissues. Cartilage specimens were minced like paste and washed three times in sterile 0.9% sodium chloride. Then, the chondrocytes were isolated with 0.25% trypsin (Difco Lab., Detroit, MI) in sterile saline for 30 minutes followed by 0.25% collagenase (Worthington, Freehold, NJ) in Dullbecco's modified Eagle's medium (SIGMA Chemical Co., St. Louise, MO) supplemented with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone (BIOWHITTAKER, Walkersville, MD) for 6 h at 37°C in a culture bottle.Finally, the isolated cells were collected by centrifugation (1,500 g, 37°C, 5 min) after removal from the culture bottle and washed three times with the culture medium. The final cell density of chondrocyte suspension was adjusted for further investigations.

2.4. Cell adhesion study

Chondrocyte adhesion to the polymer fibers was assessed by the method as previously reported by Nishimura et al. [16]. The polymer fibers of Polyglactin 910, a 90:10 copolymer of glycolide and lactide, coated with polyglactin 370 (9-0 Vicryl suture material, Ethicon Co., Somerville, NJ), were used as control materials. The fibrous samples were cut into 10 mm pieces and packed in Teflon tubes (25 mm length, 4.8 mm inner diameter) and then 0.1 ml of chondrocyte suspension containing 0.5×10^6 cells was loaded on the column at room temperature. The cells were allowed to adhere in a humidified incubator (37°C, 5% CO_2) for 1 h. Each column was gently rinsed with 1 ml of 1 M phosphate-buffered saline (PBS), and the number of unattached cells in the rinsed solution was quantified by microscopic observation using a hemocytometer. Parallel samples of $n = 5$ were used for each group of polymer fiber.

2.5. Chondrocyte culture

The 3D scaffold materials mentioned previously were used for the chondrocyte culture. Chondrcyte suspension

containing 0.6×10^6 cells was embedded on the materials. These samples were placed in a 37°C, humidified 5% CO_2 incubator for 1 h and then overlaid with 2 ml of the culture medium. Chondrocyte cultures were placed in a humidified incubator and replaced with a fresh medium twice a week.

2.6. DNA quantification

At 1, 7, and 14 days after cultivation, 5 cultured materials of each fiber group were harvested to quantify the total amount of DNA. The DNA content (μg/sample) was measured in aliquots of the sodium citrate (0.05 M phosphoric acid, 2 M NaCl) dissolved powdered samples by the modified fluorometric assay using a bisbenzididazole dye (Hoechst dye 33258, Polyscience Inc., Northampton, UK) [17]. In fluorescence measurements, disposable cuvettes were used; the excitation wavelength was 356 nm and the emission wavelength was 458 nm. The results were extrapolated from a standard curve using salmon testis DNA (Worthington, Freehold, NJ).

2.7. RNA isolation

After 14 days of culture, samples of each fiber group were frozen in liquid nitrogen prior to RNA extraction. Total RNA was extracted by the TRIspin method described previously [18]. Briefly, frozen samples were powdered in liquid nitrogen cooled with Brown Dismembrater vessels (B. Braun Biotech., Allentown, PA). The TRIzole reagent (Life Technologies, Gaitherburg, MD) was added to the powdered tissue at the rate of 1 ml/100 mg sample weight and the samples warmed to room temperature. The samples, to which chloroform was added, were centrifuged at 12,000 g for 15 min at 4°C. Then, the upper aqueous phase containing the RNA was obtained and mixed with 70% ethanol. Finally, total RNA was isolated using the RNeasy total RNA kit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol.

2.8. Semiquantitative reverse transcriptase-polymerase chain reaction (RT–PCR) analysis

Total RNA yield was fluorometrically quantified with the SYBR® Green reagent (Molecular Probes, Eugene, OR) according to the manufacturer's recommendations. According to the previous reports, semiquantitative RT–PCR analysis was performed using rabbit specific primer sets for type I collagen, type II collagen, aggrecan, and a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [19–22]. First, 1 μg of total RNA was reverse-transcribed into cDNA using the StratScript RNase H^- kit (Stratagene, La Jolla, CA). Second, aliquots (1.5 μl of 50 μl total value) of the resulting cDNA were amplified in a total volume of 50 μl containing PCR buffer, 0.2 μM dNTP mixture, 1.5 mM MgCl, 0.5 μM of each primer, and 1 unit of the Taq DNA polymerase (Life Technologies Inc., Gaithersburg, MD). As reported previously, conditions were determined to be in the linear range for both the PCR amplification and the image analysis system [19–22]. The PCR products were separated by electrophoresis of 20 μl of each reaction mixture in a 2% agarose gel at 100 V/cm in 1 × Tris-acetate-EDTA buffer. Following electrophoresis, the gels were stained with ethidium bromide, destained in distilled water and photographed using a charge coupled device (CCD) camera. Comparison to the standard 1 kb DNA ladder (Life Technologies Inc., Gaithersburg, MD) ensured proper size of PCR products. Then, the PCR products were separated by electrophoresis. Finally, the images were captured with a CCD camera and analyzed with Quantity One (PDI., Inc., Huntington Station, NY). To yield a semiquantitative assessment of the gene expression, the data were expressed as normalized ratios by comparing the integrated density values for the genes in question with those for GAPDH.

2.9. Morphological and immunohistochemical analysis

Cell morphology in the 3D scaffold material was observed by light microscopy and scanning electron microscopy (SEM) at 14 days after cultivation. At the end of the cultivation period, all samples were rinsed with Ringer's solution to remove nonattached cells. The cells in the material were fixed over night with 2.5% glutaraldehyde supplemented with 0.1 M phosphate buffer. After fixation, the SEM specimens were rinsed with 0.1 M phosphate buffer and fixed in 1% OsO_4 for 1 h, then soaked in 1% tannic acid for an additional 1 h. These processes were repeated three times. The specimens were dehydrated through a graded ethanol series and dried at the critical point of CO_2. The specimens were then mounted on an aluminum stub and sputtered with argon using an ion coater (Hitachi, Tokyo, Japan), and viewed with a SEM (Hitachi S-4500, Hitachi, Tokyo, Japan) immediately after preparation. Immunohistochemical stains were performed with anti-type I and anti-type II collagen antibodies (Fuji Pharm. Lab., Toyama, Japan) to detect expression of the type I and type II collagen products.

2.10. Statistical analysis

All data were represented as mean ± standard error. Statistical comparisons were performed using one-way analysis of variance (ANOVA) and Fisher's PLSD test. Differences were considered significant for $p < 0.05$.

3. Results

3.1. Material properties

The tensile strength of each fiber group was $87.4 \pm 2.0\,N/mm^2$ in the chitosan group, $168.2 \pm 7.0\,N/mm^2$ in the HA0.04% group, and $144.4 \pm 2.1\,N/mm^2$ in the HA0.07% group. The values of the hyaluronic acid hybrid fiber significantly increased as compared with that of the chitosan fiber ($p<0.0001$). The strain at failure was $11.2 \pm 1.7\%$ in the chitosan group, $9.8\% \pm 2.4\%$ in the HA0.04% group, and $9.7 \pm 1.9\%$ in the HA0.07% group. There were no significant differences among the 3 groups.

3.2. Cell adhesion

Adhesivities of the chondrocytes were expressed by the percentage of chondrocytes trapped in the column to the total chondrocytes loaded on the column (0.5×10^6 cells). The percentage was $53.3 \pm 7.1\%$ in the control group, $79.3 \pm 2.2\%$ in the chitosan group, 95.1 ± 1.3 in the HA0.04% group, and $90.5 \pm 3.2\%$ in the HA0.07% group. The values in the HA 0.04% and HA 0.07% groups were significantly higher than that in the control ($p<0.0001$) and in the chitosan groups ($p<0.05$).

3.3. Cell proliferation

Light micrographs (Fig. 4) showed the proliferation of chondrocytes on all the fibers at 14 days after cultivation. From day 1 to day 14 of the culture period, the total amount of DNA increased in all the fiber groups (Fig. 3). After 7days in culture, the total amount of DNA in each group was $134.5 \pm 3.9\,\mu g$/sample in the chitosan group, $142.2 \pm 11.2\,\mu g$/sample in the HA0.04% group, and $240.1 \pm 23.0\,\mu g$/sample in the HA0.07% group. The value in the HA 0.07% group was significantly higher than that in the chitosan and HA0.04% groups ($p<0.05$). On the other hand, at 14 days after cultivation, there were no significant differences among the 3 groups.

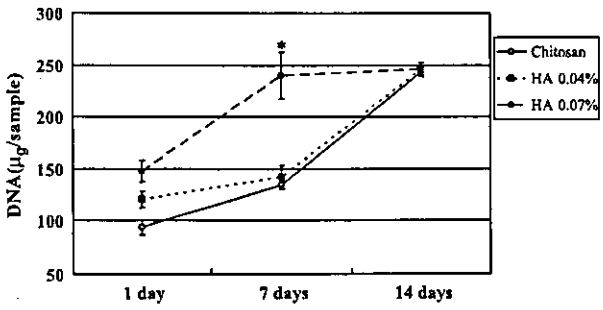


Fig. 3. The increase of DNA content per sample (μg/sample) in the chitosan and hybrid 3D scaffolds seeded with chondrocytes. $^*p<0.05$ versus the other groups. $N = 5$, mean ± standard error.

3.4. Cell morphology

There was no effect of fiber material on cell morphology at 14 days after cultivation. Light micrographs (Fig. 4) and SEM micrographs (Fig. 5A,B) revealed the characteristic round morphology of the chondrocytes on all the fibers.

3.5. Extracellular matrix products

The SEM micrographs revealed the dense fiber, which indicated the type II collagen, in interstitial space between the fibers (Fig. 5B). At 14 days after cultivation, the quantity of total mRNA yield per mg wet weight in the chitosan group, in the HA0.04% group, and in the HA0.07% group were 0.27 ± 0.02, 0.24 ± 0.02, and $0.15 \pm 0.02\,\mu g/mg$, respectively. The mRNA for GAPDH was well expressed in all samples. Based on the analysis of 1 μg of total RNA converted to cDNA by RT from each sample, integrated density values of GAPDH in the linear PCR range (25 cycles) of the chitosan group, of the HA0.04% group, and of the HA0.07% group were 0.20 ± 0.01, 0.22 ± 0.01, and 0.20 ± 0.01 integrated density units, respectively. There were no significant differences in GAPDH band density among the 3 groups. These results support the normalization of subsequent assessments to this gene. At 14 days the culture materials, normalized ratio of mRNA of type I collagen, of type II collagen, and of aggrecan to that of GAPDH is summarized in Table 1. In the values of type I and type II collagen, there were no statistically significant differences among the 3 groups (Table 1 and Fig. 6). On the other hand, no expression of the mRNA of the aggrecan was identified in the chitosan group (Table 1 and Fig. 6). The normalized ratio of the HA0.07% group significantly increased, as compared to that in the HA0.04% group ($p<0.05$). In a histochemical study, safranin O lightly stained the pericellular matrix around the chondrocytes in all the fibers at 14

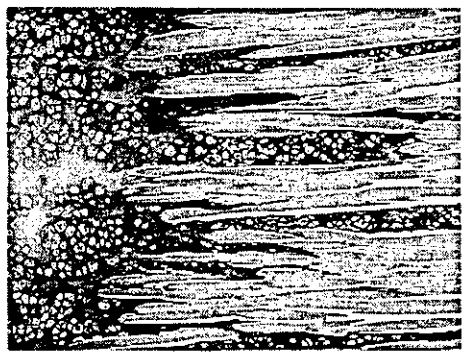
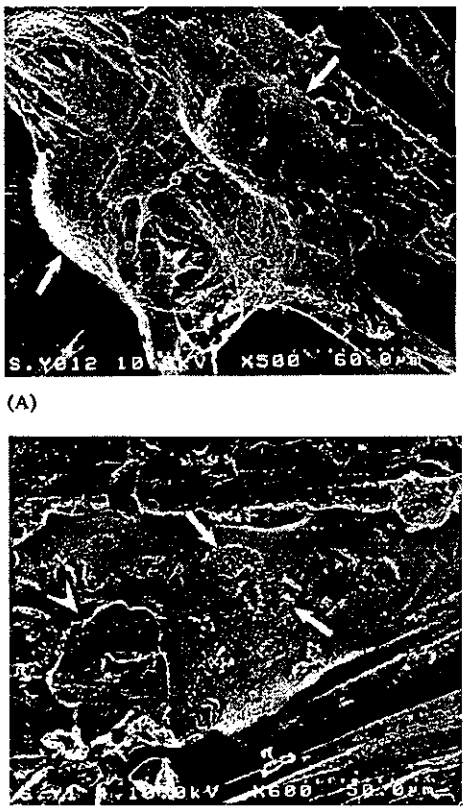


Fig. 4. Light micrograph of chondrocytes proliferated in the 3D scaffold material consisting of the chitosan-based 0.04% hyaluronic acid hybrid polymer fiber at 14 days after cultivation (hematoxylin–eosin staining, original magnification × 50).



(A)

(B)

Fig. 5. Scanning electron micrographs of the chondrocytes seeded on the 3D scaffold material consisting of the chitosan-based 0.07% hyaluronic acid hybrid polymer fiber at 14 days after cultivation. (A) Scaffold superficial surface; (B) scaffold sagittal surface. The characteristic round morphology of the chondrocytes (white arrows) and the dense fibers of the type II collagen can be found around the chondrocytes. Arrow heads, polymer fiber.

Table 1

Material groups ($n = 5$)	Type II collagen	Aggrecan	Type I collagen
Chitosan group	1.51±0.07	No expression	0.45±0.04
HA 0.04% group	1.59±0.07	1.07±0.17	0.60±0.11
HA 0.07% group	1.37±0.12	1.59±0.09*	0.43±0.11

*$p = 0.025$ versus HA 0.04% group.

days after cultivation. Immunohistochemical staining with an anti-type II collagen antibody demonstrated rich type II collagen production in the pericellular matrix (Fig. 7A). However, there was no staining for type I collagen in all the fibers (Fig. 7B).

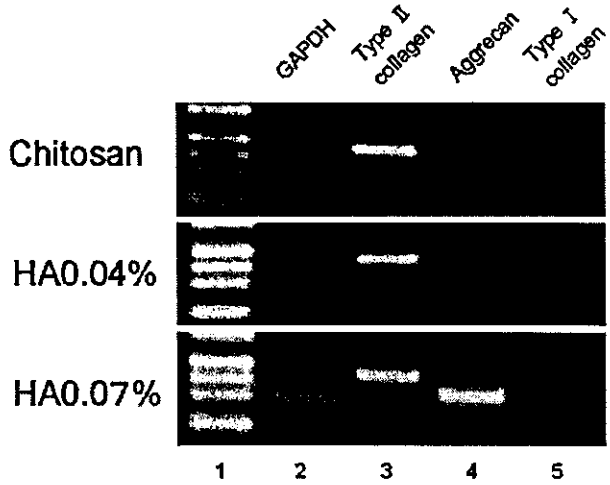


Fig. 6. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis in the chitosan, the HA0.04%, and the HA0.07% group specimens. Lane 1, ladder marker; lane 2,GAPDH; lane 3, type II collagen; lane 4, aggrecan; lane 5, type I collagen. At 14 days after cultivation, the mRNA for type II collagen was well expressed in all specimens. On the other hand, no expression of the mRNA of the aggrecan was identified in the chitosan group specimens.

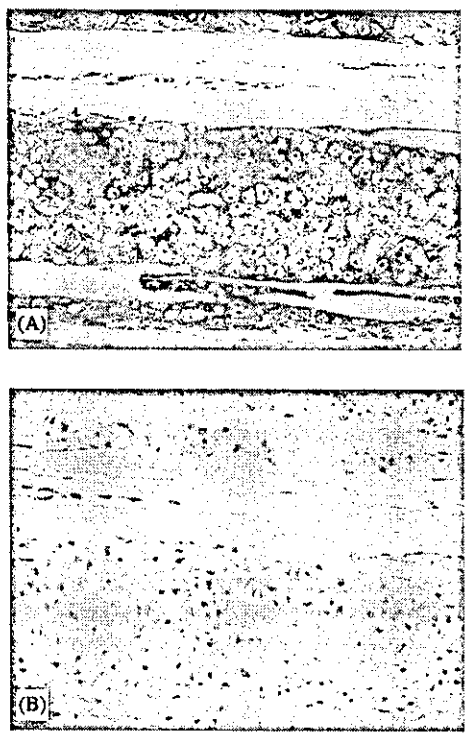


Fig. 7. Immunohistochemical stainings with anti-type I and II collagen antibodies. A, Rich production of type II collagen is indicated in the HA0.07% group. B, No staining of type I collagen is found in the HA0.07% group. (original magnification × 100).

4. Discussion

The final goal of the current study was to clarify the feasibility of the novel chitosan-based hyaluronic acid hybrid polymer fibers as a scaffold biomaterial for cartilage tissue engineering. Recently, several reports have shown the potential of chitosan scaffold biomaterial for cartilage tissue engineering [6,8,9,44]. Suh and

Matthew [9] reported that chitosan is well suited as a carrier material for the transplant of autologous chondrocytes. Our results demonstrated that adhesion, proliferation, and ECM products of the chondrocytes were significantly higher on the hybrid polymer fibers than on the non-hybrid chitosan polymer fiber, which is well accepted as a scaffold material. Based on these previous and our current results, we reasonably conclude that the chitosan-based hyaluronic acid hybrid polymer fibers have a great potential as a desirable biomaterial in cartilage tissue scaffolds.

Of emphasis in our novel biomaterial is that hyaluronic acid, which is a main component of cartilage glycosaminoglycans (GAGs), is applied to chitosan as a fundamental substance. The ideal cell-carrier substance should be one which closely mimics the natural environment in the cartilage ECM. Glycosaminoglycans, which are parts of the cartilage ECM components, play an important role in regulating expression of the chondrocyte phenotype and in supporting chondrogenesis [8,23–26]. Therefore, the application of hyaluronic acid as a component of the cartilage scaffold biomaterial must be a reasonable approach for enhancing chondrogenesis. Concerning the cell adhesivity of hyaluronic acid, Zimmerman et al. [27] showed that hyaluronic acid is an adhesion modulator molecule, which can mediate the early stage of cell-substrate interaction. On the other hand, CD44 is well known as a cell surface receptor for hyaluronic acid [28,29]. CD44 is a transmembrane glycoprotein expressed in a variety of cell types in connective tissues and a major cell surface protein in chondrocytes. This has been postulated to have a function as the principal receptor for hyaluronic acid, a common GAG component of the ECMs [29–31]. Murdoch et al. [32] demonstrated that there was a dramatic increase of CD44 expression on the isolated chondrocytes from the cartilage. Based on these previous data and the current results, we reasonably conclude that scaffold biomaterials introducing hyaluronic acid can provide excellent chondrocyte adhesive activity.

In the current study, another important point is that a polymer fiber has been applied to the scaffold biomaterial. A scaffold for cartilage tissue engineering requires adequate mechanical strength to maintain the initial shape of the implanted scaffold. Several studies have demonstrated the chondrogenic potential of GAGs-augumented chitosan hydrogels [8,45]. However, these hydrogels do not have the required mechanical strength as mentioned above. Therefore, scaffolds consisting of these hydrogels cannot be transplanted into large cartilaginous lesions in advanced degenerative diseases such as osteoarthritis and rheumatoid arthritis. To solve this drawback, we have developed a new polymer fiber as a fundamental material for 3D fabric. The obtained data showed a significant increase of mechanical strength in the hyaluronic acid hybrid fibers. This indicates that introducing hyaluronic acid to the fundamental materials plays an important role in increasing the material properties of the scaffold. Tamura et al. [15] reported the enhancement of tensile strength by the coating of alginate fiber with chitosan. Ionic interaction is the most convenient way to form a tight bond between two molecules. They concluded that the tight bond of chitosan to alginate increased the tensile strength of the hybrid fibers. In the current study, the tensile strength of a chitosan polymer fiber increased by applying hyaluronic acid coating. As chitosan is a cationic polysaccharide consisting of glucosamine residues and hyaluronic acid has anionic behavior, a tight bond between both molecules was expected. The main reason for the increase of mechanical strength in the novel hybrid fiber is this tight bond between chitosan and hyaluronic acid polymers. The novel fabric consisting of chitosan-based hyaluronic acid hybrid polymer fibers will serve as an ideal scaffold with adequate strength for cartilage tissue engineering.

Chitosan is a partially deacetylated derivative of chitin, the primary structural polymer in arthropod exoskeletons. Structurally, chitosan is a linear polysaccharide consisting of $\beta(1\rightarrow4)$ linked D-glucosamine residues with a variable number of randomly located *N*-acetyl-glucosamine groups. The average molecular weight ranges from 50 to 1000 kDa. The potential of chitosan as a biomaterial is based on its cationic nature and high charge density in solution. Madihally et al. [6] reported that the cationic nature of chitosan allowed for electrostatic interactions with anionic GAGs, PGAs, and other negatively charged species. These ionic interactions may serve as a mechanism for retaining and recruiting cells, growth factors, and cytokines within a tissue scaffold. Consequently, chitosan has been already employed as an excellent biomaterial for wound healing and tissue repair [33–35]. Since chitosan is regarded as a cationic polysaccharide showing excellent cell supporting properties, a hybrid material composed of chitosan combined with hyaluronic acid might prove to be a novel class of polyion complex effective for cartilage specific scaffolds.

The current strategies for the treatment of damaged adult articular cartilage are limited. To solve the limitations of the current operations such as osteotomies and total joint arthroplasties, several tissue engineering techniques have been developed and clinically applied to such lesions [42,43]. However, because of the mechanical weakness of scaffold materials and the limited number of donor cells, the present techniques can be used only for relatively small cartilaginous lesions following traumatic injuries and osteochondritis dessecans. Ideally, a tissue engineering technique could be available as an alternative to the current operations mentioned above for the treatment of large cartilaginous lesions

in degenerative diseases, including osteoarthritis and rheumatoid arthritis. In articular cartilage tissue engineering, we must consider that the articular cartilage is subject to excessive compression and shear stress. Therefore, to maintain the initial shape of the scaffold surface and the number of attached chondrocytes, adequate mechanical strength and highly cellular adhesivity are requirements for scaffold materials in cartilage tissue engineering. The other consideration is that the chondrocytes exhibit a profound change in their phenotype after isolation from the ECM. They show the development of a fibroblastic morphology and a switch in production from type II collagen to type I collagen. To maintain the chondrocyte phenotype through the process of cartilage regeneration, scaffold material must have the potential to support the chondrogenesis while maintaining the chondrocyte phenotype. Based on the current data, for cartilage tissue engineering, we may reasonably conclude that our novel chitosan-based hyaluronic acid hybrid polymer fiber serves as an ideal biomaterial to create a 3D fabricated scaffold with adequate strength, high cellular adhesivity, and excellent support for chondrogenesis. In the current study, we focused not on the shape or structure of the 3D fabrication for cartilage tissue, but on the development and assessment of the chitosan-based hyaluronic acid hybrid fiber as a cartilage tissue engineering scaffold. Using the novel 3D scaffold material with these properties, this tissue engineering technique would be applied to the treatment of large cartilaginous lesions in a variety of diseases such as osteoarthritis and rheumatoid arthritis.

A considerable limitation of this study is that the results were derived from an in vitro experimental model. Therefore, the biocompatibility of the current fibrous material in living joints is still unclear. A number of studies have reported the tissue response to various chitosan-based materials [36–41]. In general, these chitosan materials have been observed to evoke a minimal foreign body reaction. Sue and Matthew [9] stated that this reaction may play a role in inducing local cell proliferation and ultimately integration of the implanted material with the host tissue. However, the biocompatibility or immunological reaction of fibrous chitosan material to the joint tissue remains unclear. In addition, the process of degradation or absorption of the current fibrous material in the articular environment is still unknown. A further direction of our study will be to clarify these points using animal experimental models. Finally, further research will be needed to determine the adequate shape, pore size and mechanical properties of a 3D fabrication for cartilage tissue regeneration.

Although there is a considerable limitation as mentioned above, the data derived from this study suggest great promise for the future of chitosan-based hyaluronic acid hybrid polymer fibers as a scaffold biomaterial. The novel scaffold material will be applied to cartilage tissue engineering for relatively wide cartilaginous lesions caused by various joint diseases, including osteoarthritis and rheumatoid arthritis.

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Feasibility of Polysaccharide Hybrid Materials for Scaffolds in Cartilage Tissue Engineering: Evaluation of Chondrocyte Adhesion to Polyion Complex Fibers Prepared from Alginate and Chitosan

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The ideal cell-carrier material for cartilage regeneration should be one that closely mimics the natural environment in a living articular cartilage matrix. In the current study, we considered that alginate-based chitosan hybrid biomaterials could provide excellent supports for chondrocyte adhesion. To test this hypothesis, we investigated the adhesion behavior of rabbit chondrocytes onto an alginate polymer versus the adhesion of the chondrocytes onto some alginate-based chitosan hybrid polymer fibers in vitro. We demonstrated that the alginate-based chitosan hybrid polymer fibers showed much improved adhesion capacity with chondrocytes in comparison with alginate polymer fiber. Additionally, morphologic studies revealed maintenance of the characteristic round morphology of the chondrocyte and the dense fiber of the type II collagen produced by the chondrocytes in the hybrid polymer. On the basis of these results, we conclude that an alginate-based chitosan hybrid polymer fiber has considerable potential as a desirable biomaterial for cartilage tissue scaffolds.

Introduction

The limited potential for self-repair of articular cartilage necessitates surgical procedures to treat injured cartilage. However, no current procedures for cartilage repair have successfully regenerated long-lasting hyaline cartilage tissue to replace a cartilaginous lesion. On the other hand, a large number of tissue engineering studies, involving the regeneration of hyaline cartilage by culturing isolated chondrocytes on biocompatible and biodegradable polymers as scaffolds onto which cells are seeded, have been performed.[1−10] Some of these studies have shown the importance of selecting the appropriate biomaterials as scaffolds for the chondrocyte adhesion and supporting cell proliferation.[3,5] Although a variety of biomaterials, including both naturally occurring and synthetic, have been introduced as potential scaffolds for cartilage repair,[1,2,4,6−10] we believe that the ideal cell-carrier substance should be one that closely mimics the natural environment in the cartilage-specific extracellular matrixes (ECMs).

Given the importance of glycosaminoglycans (GAGs) in stimulating chondrogenesis in vitro,[2,8,9] the uses of GAGs or GAGs-like materials as components of a scaffold seems to be a reasonable approach for enhancing chondrogenesis. In the current study, we focused on simplifying complicated three-dimensional (3D) networks of ECMs composed of a variety of cell adhesive proteins and proteoglycans. This was achieved by preparing a novel model of polyion complex fibers from alginate and chitosan. Alginate is one of the naturally abundant and anionic polysaccharides composed of a disaccharide repeating unit, [GlcAβ (1−4) Glcβ (1−3)].[11] Moreover, this polysaccharide is known as a hyaluronic acid-like biocompatible polymer often used in biomaterials science because it contains D-glucuronic acid as a main sugar residue of the repeating unit.[12,13] Alginate could be considered to be a good mimic for cartilage GAGs. However, because of its anionic nature, the cellular adhesivity of the chondrocytes to alginate is limited.[14]

On the other hand, chitosan has been employed as an excellent biocompatible material for wound healing and tissue repair.[15−17] Since chitosan is regarded as a cationic polysaccharide showing excellent cell adhesive properties, we hypothesized that a hybrid material composed of alginate combined with chitosan could improve the chondrocyte adhesion to the material. To test this hypothesis, we investigated the in vitro adhesion behavior of rabbit chondrocytes onto

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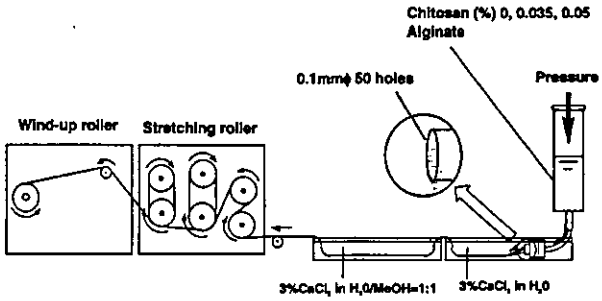

Figure 1. Original spinning apparatus for alginate fiber coated with chitosan.

alginate-based chitosan hybrid polymer fibers. The objectives of the current study are to quantify the degree of rabbit chondrocyte adhesion onto alginate-based chitosan hybrid polymer fibers and onto an alginate polymer fiber and to compare the data obtained from the respective fiber types. In addition, we present the chondrocyte proliferation and the morphologic findings onto the alginate-based chitosan hybrid polymer fibers. The results derived from this study provide the essential information on the interactions between chondrocytes and the novel hybrid polymer fibers as a biomedical material for cartilage tissue scaffolds.

Experimental Section

Preparation of Polyion Complex Fiber. The process of preparing the alginate polymer fiber and the alginate-based chitosan hybrid polymer fibers was essentially the same as that reported by coauthors.[18] A solution of sodium alginate (4% w/w, M_w = 600 000, Kibun Food Chemifa, Co. Ltd., Tokyo, Japan) and chitosan oligomer (0, 0.035, or 0.05% w/w, M_w = 18 000, Kimitsu Chemical, Co. Ltd., Tokyo, Japan) in water was poured subsequently into the coagulation system (a: 3% $CaCl_2$ in H_2O; b: 3% $CaCl_2$ in H_2O/MeOH) through a nozzle containing 50 holes, each with a diameter of 0.1 mm (Figure 1). The fibers were washed with methanol and dried at room temperature and used for the further experiments after sterilization. In the current study, alginate polymer fiber (alginate group) and alginate-based chitosan hybrid polymer fibers (alginate with 0.035% chitosan, alginate-chitosan 0.035% group; alginate with 0.05% chitosan, alginate-chitosan 0.05% group) were originally prepared. Polymer fiber of polyglactin 910, a 90:10 copolymer of glycolide and lactide, coated with polyglactin 370 and calcium stearate (9−0 Vicryl suture material, Ethicon Co., Somerville, NJ) was also used as a control material. This fibrous material, which has the same diameter as the novel fibers, has been widely used in clinical fields.

Measurement of Mechanical Properties. The mechanical properties of each fiber group were measured according to the Japanese Industrial Standards L1015. Tensile tests for five samples of each fiber group were performed at a crosshead speed of 20 mm/min using the material testing machine (P/N346-51299-02, Shimadzu, Kyoto, Japan). The cross-sectional area was determined using a microscope (BX50, Olympus, Tokyo, Japan) and a video dimension analyzer (VM-30, Olympus, Tokyo, Japan).

Microscopic Observation of Fibers. Microarchitecture of each fiber was observed by scanning electron microscopy (SEM, S-4500, Hitachi, Ltd., Tokyo, Japan). To quantitatively evaluate the alteration in the fibrous structure through the degradation process, the diameter of each fiber in wet and dry conditions (n = 10 in each group) was measured with a SEM image analysis system (Quartz PCI, Quartz Imaging Corporation, Tokyo, Japan).

Preparation of Chondrocyte Suspension. Chondrocytes were isolated from the articular surfaces of a Japanese white rabbit (2.0 kg, Hokudo Co. Ltd., Sapporo, Japan) under sterile conditions using a technique described by Wakitani et al.[19] Articular cartilage slices, gathered from the knee, hip, and shoulder joints were detached from the adherent connective tissues. The cartilage specimens were minced like paste and washed in sterile 0.9% sodium chloride. Then, the chondrocytes were isolated with 0.25% trypsin in sterile saline for 30 min followed by 0.25% type II collagenase in Dullbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B for 6 h at 37 °C in a culture bottle. Finally, the isolated cells were collected by centrifugation at 1500 g for 5 min at 37 °C after removal from the culture bottle and washed three times with the culture medium. The isolated cells were quantitated using a hemocytometer, and then the higher concentrated chondrocyte suspension was diluted to 5×10^6 cells/mL.

Cell Adhesion Study. Cell adhesion to the fibrous materials was basically assessed by the method as previously reported by Nishimura et al.[20] The fibrous samples were cut into 10 mm pieces and packed in Teflon tubes (25 mm length, 4.8 mm inner diameter, Sanplatec, Osaka, Japan). Each fibrous sample consisted of 230 fibers. Then, 0.1 mL of chondrocyte suspension containing 0.5×10^6 cells was loaded on the column at room temperature. The cells were allowed to adhere in a humidified incubator (37 °C and 5% CO_2) for 1 h. Each column was gently rinsed with 1 mL of 1 M phosphate-buffered saline (PBS) per 30 s using a syringe and the number of unattached cells was quantified by the microscopic observation of the rinsed solution. Parallel samples of n = 5 were used for each group of polymer fibers.

Cell Proliferation Study. To assess the cell proliferation on each fibrous material, the sheets consisting of each polymer fiber were created in a perpendicular pattern with one layer (8 × 8 mm). At 1, 7, and 14 days after cultivation, five cultured materials of each fiber group were harvested to quantify the total amount of DNA. The DNA content was measured in aliquots of the sodium citrate (0.05 M phosphoric acid, 2 M NaCl) dissolved powdered samples by a modified fluorometric assay using a bisbenzididazole dye (Hoechst dye 33258, Polyscience Inc., Northampton, U.K.).[21] In fluorescence measurements, the excitation wavelength was 356 nm and the emission wavelength was 458 nm. The results were extrapolated from a standard curve using salmon testis DNA (Worthington, Freehold, NJ).

Morphological Observations. Cell morphology in the fiber was observed by light microscopy and SEM after 14

Table 1. Mean Mechanical Properties in Each Fiber Group[a]

	alginate	alginate-chitosan 0.035%	alginate-chitosan 0.05%
tensile strength (N/mm^2)	242.6 ± 3.2	246.4 ± 4.1	229.0 ± 2.2[b]
strain at failure (%)	11.4 ± 0.1	11.5 ± 0.8	11.0 ± 0.2

[a] Mean ± SE. [b] $p < 0.05$ vs alginate group. $p < 0.005$ vs alginate-chitosan 0.035% group.

days in culture. For the SEM observations, the samples with chondrocyte adhesion were rinsed with Ringer's solution and then fixed with 2.5% glutaraldehyde in 0.1 M PBS. The samples were covered with 1% osmium tetroxide (OsO_4) and 1% tannic acid to fix the samples for the electron microscope. After dehydration in a graded series of ethanols starting at 50% and continuing to 100%, samples were critical-point dried with CO_2 and sputter coated with gold. Samples were visualized by SEM.

Statistical Analysis. All data were represented as mean ± standard error. Statistical comparisons among the fiber groups were performed using one-way analysis of variance (ANOVA) and Fisher's PLSD test. The alterations in the fibrous diameter between dry and wet conditions in each group were analyzed using a paired t-test. Differences were considered significant for $p < 0.05$.

Results

Mehanical Properties. Table 1 summarizes the mechanical properties in each fiber group. The tensile strength in the alginate-chitosan 0.05% group was significantly lower than that in other groups ($p < 0.05$ vs the alginate group, $p < 0.005$ in the alginate-chitosan 0.035% group). In terms of the strain at failure, there were no statistically significant differences among the three groups.

Microarchitecture of Fibers. Figure 2 shows the SEM images of fibers with altered concentrations of chitosan in dry and wet conditions. Although the surface of the alginate nonhybrid fiber was uneven, it became smooth as the chitosan concentration increased. The diameters of each fiber group in dry and wet conditions are summarized in Table 2. There were no statistically significant differences in the diameter of fiber among the three groups. No significant alterations in the values were found from dry to wet condition in any groups.

Cell Adhesion. Adhesivities of chondrocytes are expressed by the percentage of chondrocyte trapped in the column (percentage of adhesion), which is calculated using the equation given in Figure 3. As shown in Figure 3, the value in the alginate-chitosan 0.05% group was significantly higher than that in the control (83.4 ± 4.5% vs 19.2 ± 6.2%, $p < 0.0001$) and the alginate groups (83.4 ± 4.5% vs 67 ± 4.6%, $p < 0.05$).

DNA Quantification. The total amount of DNA in each fibrous material was expressed as the amount per dry weight of the cultured material ($\mu g/g$). The value at each time period is summarized in Table 3. At day 1, the value in the alginate-chitosan 0.05% group was significantly higher than that in other groups ($p < 0.01$ vs the alginate group, $p < 0.05$ vs the alginate-chitosan 0.035% group). Although there were no statistically significant differences in the DNA amount at day 7 and day 14 among the three groups, the values in the alginate-chitosan hybrid groups tended to increase from day 7 to day 14.

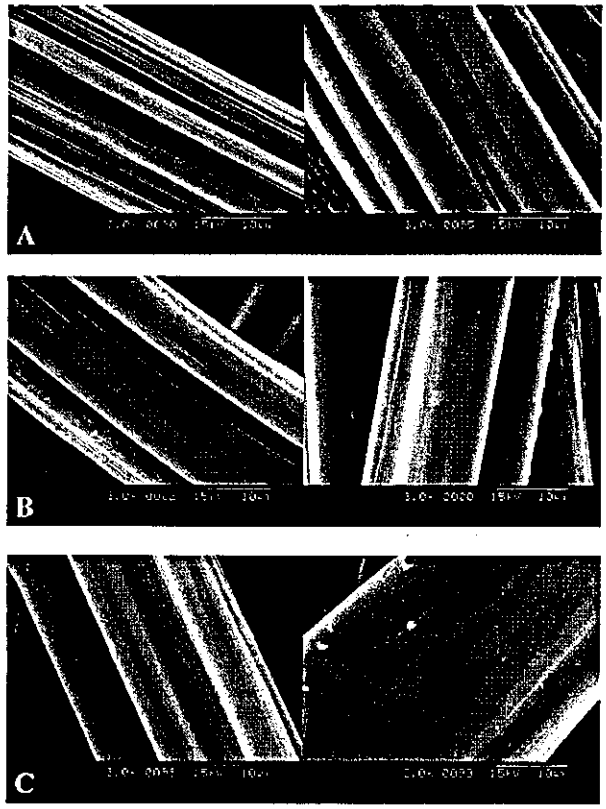

Figure 2. SEM image of fibers with altered concentrations of chitosan. A, Alginate polymer fiber; B, Alginate-based 0.035% chitosan hybrid polymer fiber; C, Alginate-based 0.05% chitosan hybrid polymer fiber. Left, dry condition; Right, wet condition. Although the surface of the alginate nonhybrid fiber is uneven, it becomes smooth as the chitosan concentration increases. No significant alterations in the microarchitecture are found between dry and wet conditions in any fiber groups.

Table 2. Mean Diameter of Each Fiber Group[a]

	alginate	alginate-chitosan 0.035%	alginate-chitosan 0.05%
dry condition (μm)	28.3 ± 1.3	27.5 ± 1.2	29.2 ± 0.8
wet condition (μm)	29.8 ± 1.7	28.6 ± 1.7	31.3 ± 1.5

[a] Mean ± SE.

Cell Morphology. Phase contrast micrographs showed the adhesion of the chondrocytes to the polymer fibers. There was no significant effect of the compositions of the polymer fiber on cell morphology after 14 days in culture. Scanning electron micrographs revealed the characteristic round morphology of the chondrocyte on an alginate-based 0.05% chitosam hybrid fiber (Figure 4). The image shows the proliferated cells in the dense fiber of the type II collagen produced by the chondrocytes (Figure 4).

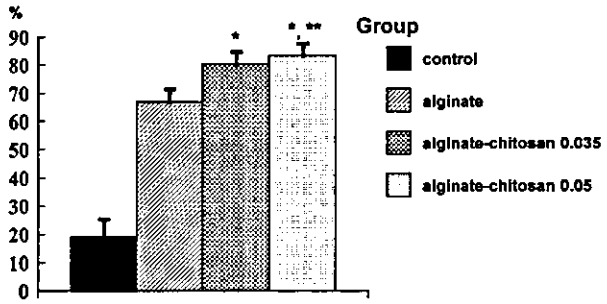

Figure 3. Percentage of chondrocytes trapped in the column (percentage of adhesion). Percentage of adhesion = (5×10^6 cells − the number of unattached cells in rinsed solution)/5×10^6 cells × 100. 0.5×10^6 cells, initial cell number in chondrocyte suspension on the column. $^*p < 0.0001$, compared with the control group. $^{**}p < 0.05$, compared with the alginate group.

Table 3. Mean Total Amount of DNA in Each Fiber Group[a]

	day 1 (μg/g)	day 7 (μg/g)	day 14 (μg/g)
alginate	19.2 ± 1.0	28.8 ± 0.8	27.5 ± 1.0
alginate-chitosan 0.035%	20.2 ± 1.2	24.6 ± 2.3	35.7 ± 5.4
alginate-chitosan 0.05%	26.2 ± 2.3[b]	26.0 ± 2.7	38.7 ± 6.2

[a] Mean ± SE. [b] $p < 0.01$ vs alginate group. $p < 0.05$ vs alginate-chitosan 0.035% group.

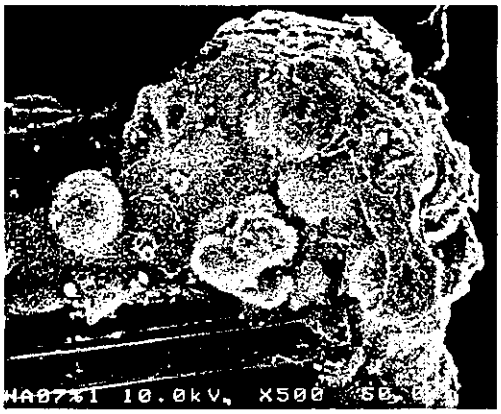

Figure 4. SEM image of rabbit articular chondrocytes seeded on alginate-based 0.05% chitosan hybrid fiber after 14 days of culture. The characteristic round morphology of the chondrocyte and the dense fiber of the type II collagen produced by the chondrocytes can be seen on the composite fibers.

Discussion and Conclusions

In articular cartilage tissue engineering, we must consider that the articular cartilage is subject to excessive mechanical stress. Therefore, to maintain the number of attached chondrocytes, highly cellular adhesivity is a requirement for scaffold materials in cartilage tissue engineering. Several studies have shown that alginate has an excellent potential of enhancing chondrogenesis in vitro culture systems.[22−24] However, one drawback of alginate is that the cells within this material do not strongly adhere to the surface due to its anionic nature. To improve the cellular adhesivity, Genes et al.[14] introduced an RGD peptide to alginate material. They demonstrated that the adhesion of chondrocytes to the RGD-alginate was 10−20 times higher than that to unmodified alginate. The background of developing the current alginate-based chitosan hybrid material is also to improve the chondrocyte adhesivity to alginate. This study demonstrated that the adhesivity of chondrocytes was significantly higher on the alginate-based chitosan hybrid polymer fiber than on the alginate polymer fiber. Madihally et al. al.[6] reported that the cationic nature of chitosan allowed for electrostatic interactions with anionic GAGs, proteoglycans, and other negatively charged species. These ionic interactions may serve as a mechanism for retaining and recruiting cells, growth factors, and cytokines within a tissue scaffold. In addition, the DNA quantification of cultured chondrocytes showed that the attached chondrocytes to the alginate-chitosan hybrid fibers tended to proliferate, as compared with those to the alginate polymer fiber. Consequently, alginate-based chitosan hybrid polymer has great potential as a desirable biomaterial for cartilage tissue scaffolds.[25−27]

Although several basic studies have demonstrated the chondrogenic potential of GAG-augmented chitosan hydrogels,[2,8,9,28,29] these hydrogels do not have the required mechanical strength. Several studies have shown a critical role of mechanical stimulations to the chondrocytes in enhancing cartilage tissue regeneration.[30−34] In vivo studies, using animal models, have clearly demonstrated that moderate exercise leads to an increase in the proteoglycan content of aricular cartilage.[31,32] In vitro studies have indicated that cyclic loading to cartilage explants stimulates synthesis of ECMs by chondrocytes.[30,33,34] In addition, Démarteau et al.[30] showed that GAGs accumulation in the chondrocyte culture system increased in response to dynamic compression only if the GAGs content prior to compression was sufficiently high. This indicates that the response of chondrocytes to dynamic compression directly depends on the GAGs amount surrounding the cells. Therefore, if the mechanical stimulation is applied to cartilage tissue engineering, a strategy for enhancing deposition of GAGs at the early culture stages must be considered. We believe that the use of alginate, which is one of the cartilage GAGs mimics, as a component of a scaffold biomaterial should be a rational strategy for cartilage tissue engineering.

A scaffold for cartilage tissue engineering requires adequate mechanical strength to maintain the initial shape of the implanted scaffold. To achieve this requirement, we have developed a new polymer fiber as a fundamental material for 3D fabricated scaffold. In the development of the novel fibers, one of the most critical points was to increase the mechanical strength of the fibers for 3D fabrication. The obtained mechanical properties of the novel fibers indicated enough mechanical strength to create a 3D fabricated scaffold using the original apparatus. On the other hand, Silver et al.[35] reported that the tensile strength of the type II collagen fibrous tissue in the cartilage was between 1000 N/mm^2 and 3000 N/mm^2. In the current study, the tensile strength of the fiber was 242.6 N/mm^2 in the alginate group, 246.4 N/mm^2 in the alginate-chitosan 0.035% group, and 229.0 N/mm^2 in the alginate-chitosan 0.05% group. Theoretically, the mechanical property of a scaffold material consisting of a large number of the current fibers should be comparable to that of the type II collagen fibrous tissue. Therefore, a 3D fabricated scaffold based on the novel fibers

may have suitable mechanical strength for cartilage tissue regeneration.

Regarding technical limitations in preparing the novel fiber, we must consider the following point: alginate is an anionic polysaccharide consisting of polyguluronate and polymannuronate residues. Its is well-known that the calcium ion chelates to alginate chains of polyguluronate sequences, and the strength and specificity of the complexes are explained in terms of an egg-box model.[36] In contrast, chitosan is a cationic polysaccharide consisting of glucosamine residues. The calcium ion and chitosan exist in the first coagulation bath. The strength of alginate fibers would decrease due to the inhibition of chelation of calcium ion with alginate chains at high concentration of chitosan. Therefore, in this study, we did not prepare the hybrid fibers coated with over 0.05% chitosan concentration.

In conclusion, the alginate-based chitosan hybrid polymer fibers promoted favorable biological responses of seeded chondrocytes including enhanced cell attachment and proliferation in vitro. In addition, the mechanical strength of these fibers could make it possible to create a 3D fabricated scaffold using an apparatus formed from the novel fibers. Ideally, the mechanical properties of 3D scaffolds based on the alginate-based chitosan hybrid fibers must be tested to determine the optimum properties for cartilage regeneration. However, the data obtained here strongly suggest that the novel fibers may be a suitable candidate for 3D scaffold biomaterial in cartilage tissue engineering. Further studies will be performed to design a desirable 3D fabricated scaffold for cartilage regeneration based on the novel fibers, to clarify the biodegradability and biocompatibility in living joints, and to evaluate the in vivo cartilage regeneration using the developed 3D scaffold.

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当科における関節リウマチに対する工藤式 type 5 人工肘関節置換術の治療成績

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Key Words : Rheumatoid arthritis（慢性関節リウマチ），Kudo elbow（工藤式人工肘関節），
Total elbow arthroplasty（人工肘関節置換術）

【目的】RA による肘関節障害に対する Kudo Elbow Type 5 人工肘関節置換術の治療成績を検討した．

【方法】対象は 1994 年以降，Kudo Elbow Type 5 を使用し，手術を施行した 52 肘のうち，2 年以上経過観察し得た 20 例・25 肘，男性 3 例・女性 17 例，手術時年齢は平均 61 歳であった．Larsen 分類では stageIV:18 肘，stage: Vは 7 肘であった．手術手技は Kudo の方法 [2]に準じ Campbell の後方アプローチにて進入，尺骨神経は皮下前方移行を行った．後療法は術後 2 週間肘関節 70° 屈曲位でのシーネ固定を行い，術後 4 週間は夜間シーネ固定とした．上腕骨コンポーネントはセメント固定 2 肘・セメントレス固定 23 肘であった．尺骨コンポーネントは metal back type・セメント固定 6 肘，metal back type・セメントレス固定 3 肘，残りの 16 肘は all HDP type・セメント固定とした．経過観察期間は平均 4 年 11 ヵ月であった．臨床評価は，ROM・Mayo Elbow Performance Score [3] を用いた．X 線学的評価では各コンポーネントの上腕骨・尺骨に対する人工関節の設置角度・clear zone と loosening の有無について検討した．Clear zone はコンポーネントおよびセメント周囲の 2 ㎜以下の radiolucent line とし，2 ㎜より幅広い radiolucent line の存在や明らかなコンポーネントの転位は loosening と判定した．また術中・術後の合併症の有無についても調査した．

【結果】肘関節平均 ROM は，伸展は術前後とも–35° と伸展制限が残存したが，屈曲は術前 103° から 134°，回内は 52° から 65°，回外は 60° から 73° と改善された（図 1）．Mayo Score は 20 例で調査可能であり，術前 39 点から術後 89 点と有意に改善した．なかでも疼痛と日常生活動作で著しい改善を認めた（図 2）．

コンポーネント設置角度は，正面像で上腕骨コンポーネントは平均 2.5° 内反，側面像では平均 0.3° 前傾していた．尺骨コンポーネントは平均 9.1° 尻上がりとなった（図 3）．上腕骨コンポーネントに clear zone は認められなかった．X 線側面像での尺骨コンポーネント周囲の clear zone は 25 肘中 8 肘（全例滑車切痕部），loosening は 1 肘（尺骨コンポーネントステムに及ぶ clear zone）に認められた．Clear zone の層別内訳は尺骨コンポーネントとセメント間 8 肘，セメントと尺骨間 1 肘であった．

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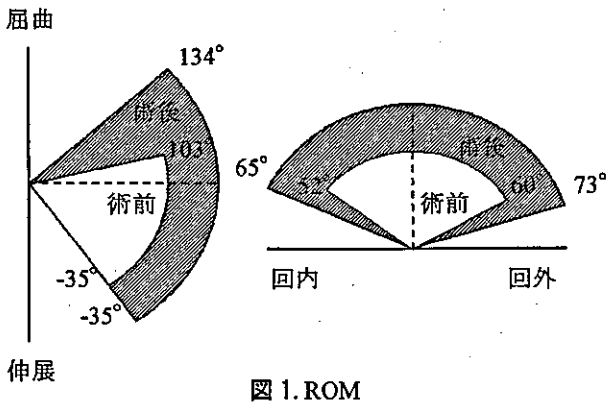

図 1. ROM

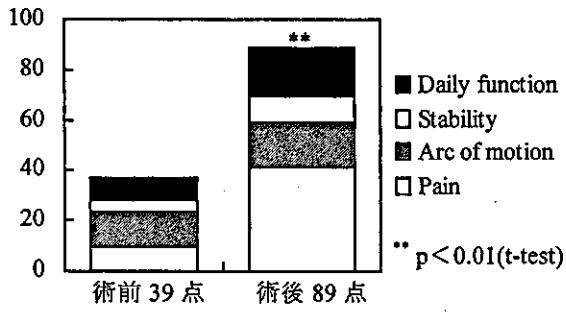

図 2. Mayo Elbow Performance Score

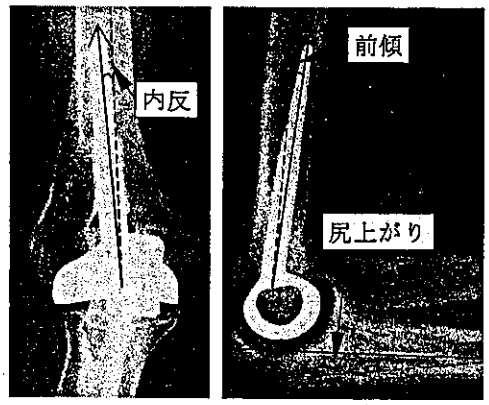

図 3. コンポーネント設置角度

25 肘中 4 肘（16%）で合併症が認められた．術中合併症として，上腕骨内顆骨折を 1 例に生じ，骨接合術を追加した．術後合併症としては，術後早期の脱臼・縫合部皮膚欠損・肘頭骨折がそれぞれ 1 例ずつ認められた．これらに対する対処法として，脱臼に対しては上腕三頭筋

と内側側副靭帯再建術を，縫合部皮膚欠損に対しては rotational flap を追加した．また clear zone が生じた 1 肘では術後軽微な外力による肘頭骨折を生じたため，骨接合術を施行したが骨癒合が得られず，最終的に尺骨コンポーネント再置換とプレートによる骨接合術を行った．

【症例】69 歳・女性．術前の肘関節 ROM は伸展-60°，屈曲 150° で，強い疼痛を伴った不安定性があり，ADL 上支障があったため，右人工肘関節置換術を施行した．術前 X 線所見では Larsen 分類 grade 4 と判断した．Mayo Score は 60 点，Fair であった（図 4）．術後 2 年 3 ヵ月時，X 線上 clear zone は認められるものの，伸展-30° ・屈曲 150°，Mayo Score も 100 点・Excellent に改善した（図 5）．

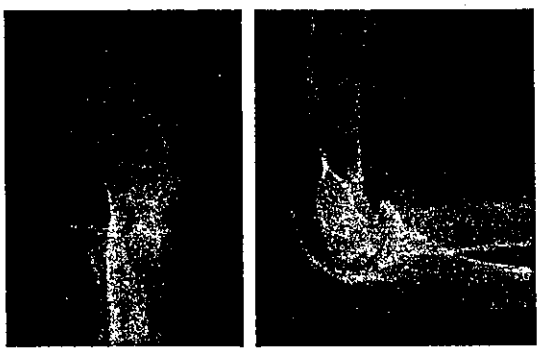

図 4. 術前 X 線

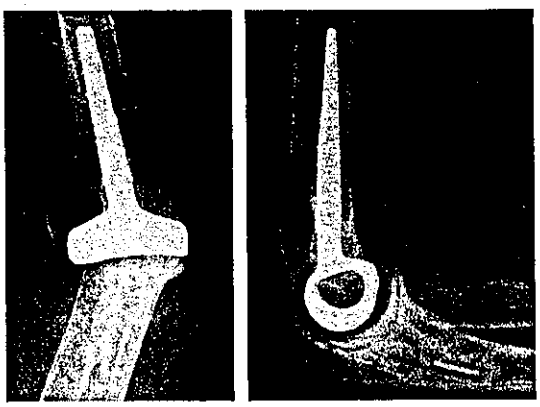

図 5. 術後 X 線

【考察】多関節障害のみられる RA 患者では肩関節，肘関節，手・指関節の変形，可動域制限や疼痛により，ADL は著しく制限されている．このような患者の中には肘関節障害が上肢機能障害の主因となっていることも多く，手術による肘関節機能の改善が上肢機能・ADL に与える効果は非常に大きいと考えられる．

当科では X 線所見上 Larsen 分類 grade 4 以上の関節破壊が進行した症例に対して，肘関節障害の改善を目的とし，人工肘関節置換術を行っている．そのなかでも，不安定性が比較的軽度で，骨欠損の少ない症例に対しては，表面置換型の Kudo Elbow を使用し，1994 年以降は安定した結果が報告されている Kudo Elbow type 5 を用いている．

今回の調査では ROM において，屈曲は吉野ら[4]の述べる機能的可動域を獲得することができたが，術後も伸展制限は残存した．表面置換型では骨切除量をできるだけ少なくするために関節部分で延長が起こってしまうこと，前方軟部組織の拘縮，肘頭先端や肘頭窩の骨棘・セメントの遺残，尺骨コンポーネントの尻上がり設置がこの要因として考えられている[1]．我々の症例においても，尺骨コンポーネントは平均 9.1° 尻上がりに設置されており，このことは伸展制限が残存した要因の一つであったと考えている．

臨床所見では Mayo Elbow Performance Score の全ての項目において改善がみられたが，特に疼痛・日常生活動作項目における改善が大きく，著明な除痛効果・屈曲域の拡大による患者の満足度は高かった．

X 線上の clear zone・loosening の発生頻度に関しては，報告者によりさまざまであるが，我々の症例では 25 肘中 8 肘（32%）に clear zone が，1 肘（4%）で loosening が認められた．最も早いものでは術後 2 年でセメント周囲に clear zone が出現した症例もあったが，最終経過観察時では clear zone の拡大や臨床症状の悪化は認められず，clear zone がすぐに loosening に結びつくものではないと考えている．しかしながら，1 例ではあるが loosening が生じた後に，軽微な外力によって肘頭骨折を来たした症例もあり，X 線上変化が認められた症例については注意深い経過観察が必要であると思われた．

Clear zone・loosening が認められた 9 例において尺骨コンポーネントの種類・固定法に関して検討したところ，9 肘中 7 肘が all HDP type・セメント固定であり，all HDP type・セメント固定例では clear zone を生じる傾向にあると考えられた．

【まとめ】RA 肘に対して工藤式人工肘関節置換術を行い，中期経過観察期間ではあるが臨床上満足のいく結果を得た．長期成績の追跡が必要ではあるが，本法は肘関節機能を改善し得る有用な治療法であると考えられた．

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PALMAR DISLOCATION OF THE METACARPOPHALANGEAL JOINT OF THE FINGER

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We report a case of palmar dislocation of a finger metacarpophalangeal joint. Disruption of all the supporting structures of this joint and rupture of the flexor tendon sheath caused marked instability. Treatment was by open reduction and repair of the collateral ligaments.
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Although dorsal dislocations of the metacarpophalangeal joint are well documented (Green and Terry, 1973; Kaplan, 1957), palmar dislocations of this joint are rare.

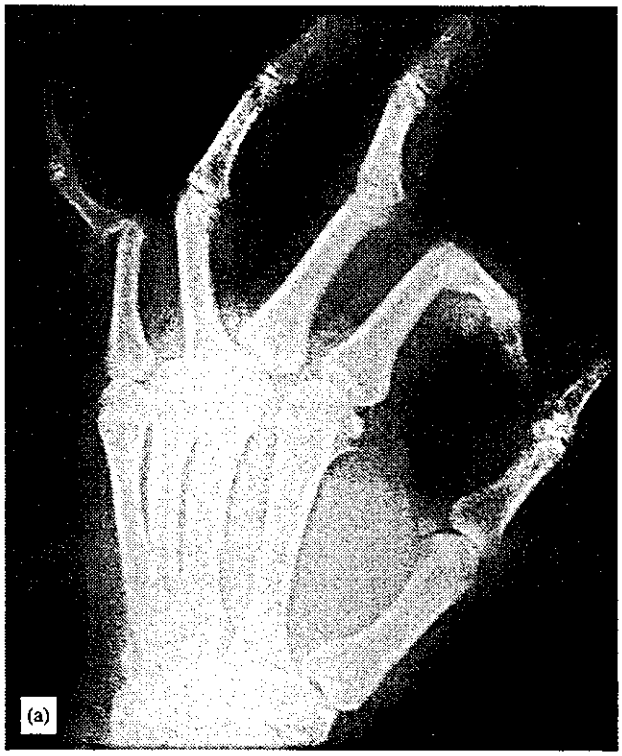
CASE REPORT

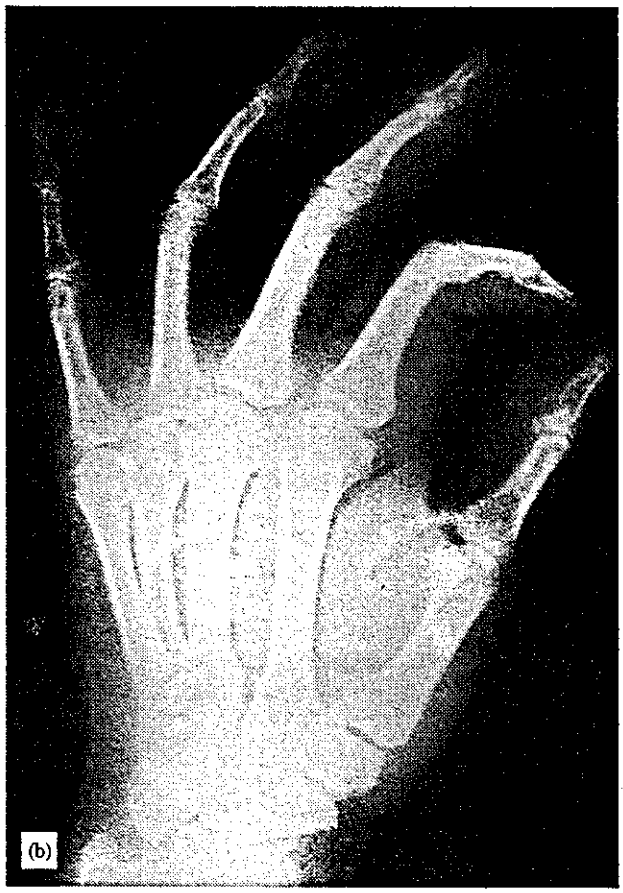
A 52-year-old woman suffered an injury to her left hand when she was dragged with great force by a dog while holding the dog leash in her left hand. Dorsal dislocations of the proximal interphalangeal joints of the middle and little fingers, and a palmar dislocation of the metacarpophalangeal joint of the ring finger were diagnosed (Fig 1). The proximal interphalangeal joints were successfully reduced under local anaesthesia, but reduction of the metacarpophalangeal joint failed. She was referred to us, 6 days later and an open reduction was attempted. Through a dorsal incision, the extensor hood was incised radial to the extensor tendon. The base of the proximal phalanx was found palmarly dislocated, and the flexor tendon lay radial to the joint (Fig 2). Both of the collateral ligaments were torn, and the dorsal capsule was avulsed from the dorsal aspect of the base of the proximal phalanx. There was a small chondral defect on the dorsoulnar aspect of the metacarpal head. The metacarpophalangeal joint could be reduced manually, but it was unstable, and readily redislocated palmarly. A palmar incision was then made, revealing a rupture of the flexor tendon sheath and avulsion of the palmar plate from the base of the proximal phalanx (Fig 3). The base of the proximal phalanx protruded on the ulnar side of the flexor tendon into the subcutaneous layer. The radial collateral ligament was mainly avulsed from its distal, and the ulnar collateral ligament from its proximal, attachment. Both collateral ligaments were repaired with suture anchors (Micro Anchor, Mitek, Norwood, MA), stabilizing the joint (Fig 4). The extensor hood was repaired and the finger was immobilized with the metacarpophalangeal joint in 30° flexion in a dorsal splint for 2 weeks. Active range of motion exercises were then started. Eight months after surgery the metacarpophalangeal joint flexed to 70° and the extension was only limited by 10°. The patient experienced no pain or disability in any of her daily activities.

DISCUSSION

Palmar dislocation of the metacarpophalangeal joint of a finger is a rare injury. Since the first description by McLaughlin (1965), only 15 cases have been reported in English language literature (Table 1), and its pathogenesis remains uncertain (Hargarten and Hanel, 1992; Lam et al., 2000). Wood and Dobyns (1981) reported three cases, and reproduced the injury in five of ten cadaver specimens by applying hyperflexion and a proximal translational force to the proximal phalanx. They concluded that avulsion of the dorsal capsule from the metacarpal neck and its interposition into the metacarpophalangeal joint as a result of a hyperflexion injury were the essential pathologic processes in the injury. Renshaw and Louis (1973), however, stated that the mechanism of injury is forceful hyperextension of the metacarpophalangeal joint, followed by distal attachment avulsion of the palmar plate which is subsequently entrapped in the palmarly dislocated metacarpophalangeal joint. However, they could not reproduce either disruption of the palmar plate or the palmar dislocation in any of 16 cadaver specimens. Betz et al. (1982) suggested that a combination of active flexion and forceful hyperextension was important in producing a palmar dislocation, and stated that the lack of an active flexion component explained why it had not been possible to produce palmar dislocations in cadavers. Rotatory force was thought to be responsible for the ruptures of the collateral ligaments that occurred in four of the 15 previously reported cases (Betz et al., 1982; Mlsna et al., 1993; Moneim, 1983; Wood and Dobyns, 1981).

From the history obtained from our patient, the main cause of the injury in our case was probably a hyperextension force applied during strong active flexion of the digit. The chondral defect at the dorsoulnar aspect of the metacarpal head and the rupture of the palmar plate also suggest that a





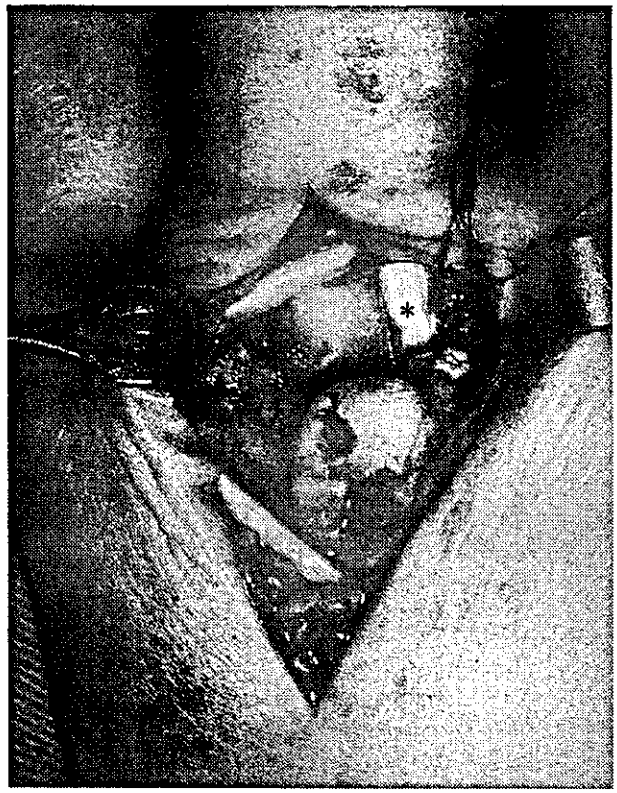


Fig 2 Intraoperative photograph through the dorsal incision. The base of the proximal phalanx is palmarly dislocated, and the flexor tendon (asterisk) lies radial to the metacarpophalangeal joint. There is a small chondral defect at the dorsoulnar aspect of the metacarpal head (arrow head).

hyperextension injury was the mechanism of dislocation. The rupture of the collateral ligaments may indicate that forceful rotation occurred during the dislocation.

Reduction in our case was not difficult, but the joint easily dislocated palmarly until the collateral ligaments were repaired.

The rupture of the flexor tendon sheath as well as the ruptures of the palmar plate and the collateral ligaments may explain the palmar instability. If the tendon sheath is not intact, the flexor tendons run more palmarly than normal when the metacarpophalangeal joint is flexed and this makes their moment arm greater than that of the extensors. In an unstable metacarpophalangeal joint which has no support from the collateral ligaments, the palmar plate or the joint capsule, this imbalance of the flexor–extensor mechanism may make the joint unstable in the palmar direction. This is why maintenance of the reduced position was difficult in our case.

Fig 1 a) Radiograph of the initial injury. Dorsal dislocations of the proximal interphalangeal joints of the middle and little fingers and a palmar dislocation of the metacarpophalangeal joint of the ring finger. b) after reduction of proximal interphalangeal joints.