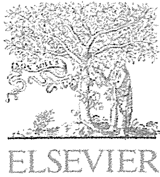
2011年4月	第50回日本生体医工学会大会	深部組織の高分解能画像化に向けた光音響画像診断法の開発と評価	平沢壮, 石原美弥, 辻田和宏, 入澤覚, 北垣学, 藤田真敬, 菊地眞
2011年3-4月	CiRA国際シンポジウム	Development of methods to inhibit tumorigenesis after transplantation of differentiated iPS cells	Matsumura K, Ishihara M, Ichiki Y, Arai M, Ishihara M, Kobayashi Y, Kikuchi M
研究分担者　加藤玲子			
2012年3月	日本薬学会 第132年会	A549細胞を用いたナノマテリアルの in vitro生体影響評価系の検討	宮島敦子, 酒井恵子, 河上強志, 加藤玲子, 松岡厚子, 尾崎正康, 宇佐見誠, 伊佐間和郎
2012年3月	The 51st Annual Meeting of the Society of Toxicology	Cytotoxicity studies in A549 cells cultured on 2-methacryloyloxyethyl phosphorylcholine polymers	Miyajima-Tabata A, Kato R, SakaiK, Okada E, Matsuoka A
2012年3月	The 51st Annual Meeting of the Society of Toxicology	Comparison of protein expression profiles in human mesenchymal stem cells cultured on surface-modified titanium with chemical treatments	Kato R, Haishima Y, Hasegawa C,Matsuoka A
2011年11月	第33回日本バイオマテリアル学会	チタン系金属, 合成高分子等の医用材料上で培養したCHL細胞の細胞毒性および遺伝毒性	宮島敦子, 加藤玲子, 酒井恵子, 松岡厚子
2011年11月	第33回日本バイオマテリアル学会	異なる表面処理を施したチタンプレート上で培養したヒト間葉系幹細胞のタンパク質発現解析	加藤玲子, 配島由二, 長谷川千恵, 松岡厚子

Ｖ．研究成果の刊行物・別刷

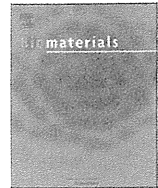
Biomaterials 33 (2012) 3846–3851



Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials



Cartilage repair in transplanted scaffold-free chondrocyte sheets using a minipig model

Goro Ebihara [a], Masato Sato [a,*], Masayuki Yamato [b], Genya Mitani [a], Toshiharu Kutsuna [a], Toshihiro Nagai [a], Satoshi Ito [a], Taku Ukai [a], Miyuki Kobayashi [a], Mami Kokubo [a], Teruo Okano [b], Joji Mochida [a]

[a] *Department of Orthopaedic Surgery, Surgical Science, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan*
[b] *Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan*

ARTICLE INFO

Article history:
Received 19 January 2012
Accepted 31 January 2012
Available online 25 February 2012

Keywords:
Cartilage tissue engineering
Chondrocyte
Cell culture
Animal model
Transplantation

ABSTRACT

Lacking a blood supply and having a low cellular density, articular cartilage has a minimal ability for self-repair. Therefore, wide-ranging cartilage damage rarely resolves spontaneously. Cartilage damage is typically treated by chondrocyte transplantation, mosaicplasty or microfracture. Recent advances in tissue engineering have prompted research on techniques to repair articular cartilage damage using a variety of transplanted cells. We studied the repair and regeneration of cartilage damage using layered chondrocyte sheets prepared on a temperature-responsive culture dish. We previously reported achieving robust tissue repair when covering only the surface layer with layered chondrocyte sheets when researching partial-thickness defects in the articular cartilage of domestic rabbits. The present study was an experiment on the repair and regeneration of articular cartilage in a minipig model of full-thickness defects. Good safranin-O staining and integration with surrounding tissues was achieved in animals transplanted with layered chondrocyte sheets. However, tissue having poor safranin-O staining—not noted in the domestic rabbit experiments—was identified in some of the animals, and the subchondral bone was poorly repaired in these. Thus, although layered chondrocyte sheets facilitate articular cartilage repair, further investigations into appropriate animal models and culture and transplant conditions are required.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Articular cartilage is hyaline cartilage featuring an extracellular matrix (ECM) consisting of an intricate collagen network and proteoglycans, and is highly resistant to mechanical loads. However, lacking a blood supply and having a low cellular density, articular cartilage has a minimal potential for self-repair. Therefore, wide-ranging cartilage damage rarely resolves spontaneously. Left untreated, cartilage damage in a load-bearing area causes secondary degeneration of the surrounding cartilage and ultimately exerts negative effects on routine activities. Numerous treatments have been developed to repair articular cartilage damage, typical examples being chondrocyte transplantation [1], mosaicplasty [2], and microfracture [3]. These treatments have produced satisfactory outcomes. However, the literature also documents treatment failures [4–8].

Autologous chondrocyte implantation (ACI), first reported by Brittberg et al. [1], has been performed over 20,000 times worldwide. However, two sites for the resection of cartilage and periosteum must be sacrificed to repair a single cartilaginous lesion. This is the largest drawback of ACI. Other problems include periosteal hyperplasia and incompatibility with surrounding tissues [5]. Problems associated with mosaicplasty include a limited number of donors of healthy cartilage and the need for long-term monitoring for damage at the harvest sites [2]. Microfracture induces cartilage regeneration by promoting the migration of mesenchymal cells from the marrow, but results in the production of fibrocartilage, which is mechanically weaker than hyaline cartilage [9].

Previously, we prepared highly adhesive layered chondrocyte sheets without a scaffold and with a short culture time using a temperature-responsive culture dish (Fig. 1a, b). On transplantation, these layered chondrocyte sheets suppressed degeneration in articular cartilage [10–12]. These temperature-responsive culture dishes have already been applied to research in various fields of regenerative medicine, including the regeneration of myocardium [13,14], vascular epithelium [15], cornea]16], hepatocytes [17], and renal cells [18]. This method has also been applied

* Corresponding author. Tel.: +81 463 93 1121; fax: +81 463 96 4404.
E-mail address: sato-m@is.icc.u-tokai.ac.jp (M. Sato).

0142-9612/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2012.01.056

Author's personal copy

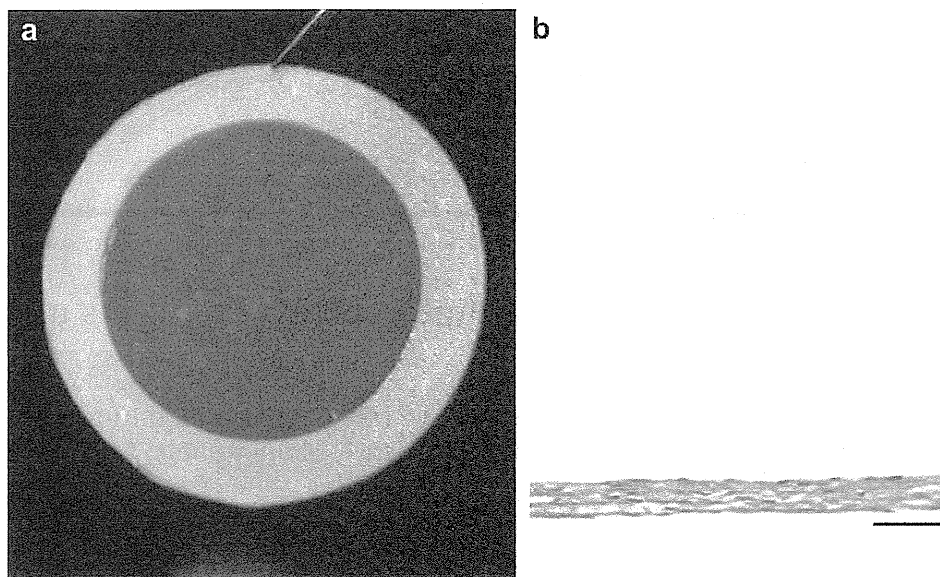


Fig. 1. (a) Layered chondrocyte sheet. Chondrocytes cultured on a temperature-responsive surface can be released from the dish (4.2 cm^2) by reducing the temperature without the need for proteolytic enzymes. Confluent cultured chondrocytes were harvested as a single contiguous cell sheet retaining cell–cell junctions, and extracellular matrix (ECM) was deposited on the basal side. (b) Chondrocyte sheets can be layered and thereafter adhere to other cell sheets. Culture of five-layered chondrocyte sheets can be continued for up to 1 week (scale bar = 150 μm).

clinically to the myocardium and cornea [16]. The surface of a temperature-responsive culture dish is coated with a polymer (poly(*N*-isopropylacrylamide)), which becomes hydrophilic or hydrophobic in a reversible manner, depending on the temperature [19]. The polymer has a low critical solution temperature of 32 °C, below which it becomes soluble in water. Based on this characteristic, the temperature-responsive culture dish has a weakly hydrophobic surface, similar to that of commercially available dishes, so it can be used to culture cells in a conventional manner when the temperature is 37 °C or higher. However, the surface of the dish becomes hydrophilic when the temperature falls below the critical solution temperature. Therefore, confluent sheets of cultured cells can be released spontaneously from the hydrophilic dish surface by reducing the temperature to below 32 °C [20]. Using this method, cultured cells can be harvested as a sheet without damaging cell–cell junctions and the ECM because it eliminates the need for conventional enzymatic harvesting with trypsin. Such cell sheets have been reported to have various advantages, including preservation of the normal phenotype and expression of adhesion proteins on the sheet base [21]. Furthermore, these cell sheets can also be superimposed to prepare layered 'tissue' because the ECM is preserved on the base, and such three-dimensional (3D) manufactured tissue has already been used successfully in transplantation [14]. We confirmed previously that such layered chondrocyte sheets are able to maintain a normal chondrocyte phenotype in the knee joints of rabbits. Moreover, they can be attached to injured cartilage, thereby acting as a barrier to prevent the loss of proteoglycan from these sites, while also protecting them from catabolic factors [10].

The objective of this study was to investigate the ability of layered chondrocyte sheets to repair and regenerate tissue in a minipig-based large animal model of full-thickness defects of articular cartilage.

2. Materials and methods

All animal experiments were approved and carried out following the Guidelines of Tokai University on Animal Use.

2.1. Temperature-responsive culture dishes

Specific procedures for the preparation of temperature-responsive culture dishes (provided by CellSeed, Tokyo, Japan) were as described [19]. Briefly, *N*-isopropylacrylamide (IPAAm) monomer solution was spread onto commercial tissue culture polystyrene dishes. These dishes were then subjected to electron beam irradiation, thus resulting in polymerization and covalent bonding of IPAAm to the dish surface. Poly-IPAAm (PIPAAm)-grafted dishes were rinsed with cold distilled water to remove ungrafted IPAAm. The dishes were then sterilized using ethylene oxide gas [22].

2.2. Chondrocytes from minipigs and proliferation on a temperature-responsive surface

Five minipigs aged 7–8 months and weighing 21.3–21.5 kg were used as the source of chondrocytes. Cartilage samples were collected from the femoral compartment of the knee joint and subjected to enzymatic processing; they were then seeded and cultured according to the method of Sato et al. [23]. Briefly, chondrocytes were digested for 1 h in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Gibco, Grand Island, NY, USA) containing 0.4% Pronase E (Kaken Seiyaku Inc., Tokyo, Japan) and for 4 h further in DMEM/F12 containing 5 mg/ml collagenase type 1/CLS1 (Worthington Inc., Lakewood, NJ, USA) at 37 °C in an atmosphere of 5% CO_2 and 95% air. Digested tissue was passed through a Falcon cell strainer (BD Biosciences, Franklin Lakes, NJ, USA) with a pore size of 100 μm. Cells were then seeded at high density (50,000 cells/cm^2) into temperature-responsive dishes (4.2 cm^2; provided by CellSeed, Tokyo, Japan) and were cultured in DMEM/F12 supplemented with 20% fetal bovine serum (FBS; Gibco), 50 μg/ml ascorbic acid (Wakojunyakukougyou Corp., Osaka, Japan) and 1% antibiotic–antimycotic mix (Gibco) at 37 °C in an atmosphere of 5% CO_2 and 95% air for a week. Culture dishes were removed from the incubator when the cells reached confluence and were left to stand at 25 °C for 30 min. After the culture medium had been removed, cell sheets were harvested as described by Kaneshiro et al. [10].

2.3. Measurement of chondrocyte proliferative activity

Chondrocytes isolated and cultured as above (3.0×10^4 cells) were cultured on 24-well plates and cell proliferation activity was measured using a thiazolyl blue tetrazolium bromide (MTT) assay on days 3, 5 and 7. MTT (Dojindo, Kumamoto, Japan) was added to each well of the 24-well plate and incubated for 2 h at 37 °C in the dark. The resulting crystals were solubilized in dimethyl sulfoxide. Absorbance was read using an enzyme-linked immunosorbent assay (ELISA) plate reader at 590 nm, with absorbance as a function of viable cell number. Data are expressed as the mean ± standard error of the mean.

Table 1
Variables used in the International Cartilage Repair Society (ICRS) grading system and the ICRS remodeling system for evaluating subchondral bone maintenance.

Variable	Comment
Ti:	**Tissue morphology** 4 = Mostly hyaline cartilage 3 = Mostly fibrocartilage 2 = Mostly noncartilage 1 = Exclusively noncartilage
Matx:	**Matrix staining** 1 = None 2 = Slight 3 = Moderate 4 = Strong
Stru:	**Structural integrity** 1 = Severe integration 2 = Cysts or disruptions 3 = No organization of chondrocytes 4 = Beginning of columnar organization of chondrocytes 5 = Normal, similar to healthy mature cartilage
Clus:	**Chondrocytes clustering in implant** 1 = 25–100% of cells clustered 2 = <25% of the cells clustered 3 = No clusters
Tide:	**Intactness of calcified cartilage layer, formation of tidemark** 1 = <25% of the calcified cartilage layer intact 2 = 25–49% of the calcified cartilage layer intact 3 = 50–75% of the calcified cartilage layer intact 4 = 76–90% of the calcified cartilage layer intact 5 = Complete intactness of the calcified cartilage layer intact
Bform:	**Subchondral bone formation** 1 = No formation 2 = Slight 3 = Strong
SurfH:	**Histologic appraisal of surface architecture** 1 = Severe fibrillation or disruption 2 = Moderate fibrillation or irregularity 3 = Slight fibrillation or irregularity 4 = Normal
FilH:	**Histologic appraisal defect filling** 1 = <25% 2 = 26–50% 3 = 51–75% 4 = 76–90% 5 = 91–100%
Latl:	**Lateral integration of implanted material** 1 = Not bonded 2 = Bonded at one end/partially both ends 3 = Bonded at both sides
Basl:	**Basal integration of implanted material** 1 = <50% 2 = 50–70% 3 = 70–90% 4 = 91–100%
InfH:	**Inflammation** 1 = No inflammation 3 = Slight inflammation 5 = Strong inflammation
Hgtot	**Histologic grading system** Some of the histologic variables: tissue morphology (Ti), matrix staining (Matx), structural integrity (Stru), cluster formation (Clus), tidemark opening (Tide), bone formation (Bform), histologic surface architecture (SurfH), histologic degree of defect filling (FilH), lateral integration of defect filling tissue (Latl), basal integration of defect filling tissue (Basl) and histologic signs of inflammation
Remod:	**Subchondral bone remodeling (loose textures of highly cellular tissue composed mostly of fibroblasts)** 1 = No remodeling 2 = Discrete cellularity 3 = Moderate cellularity 4 = High cellularity

Presentation of the ICRS histological grading system (Hgtot) and remodeling scores (Remod) based on a modified ICRS grading scale [30] developed by O'Driscoll et al. [31] In Hgtot, 11 histologic categories were evaluated and scored. The total score ranged from 11 points (no repair) to 45 points (normal articular cartilage).

2.4. Transplantation of chondrocyte sheets

Before the surgery of implantation, 0.2 mg/kg dormicum (Midazolam 5 mg/1 ml, Astellas Pharma, Tokyo, Japan) and 40 μg/kg medetomidine (Domitor 1 mg/ml, Meiji Seika Pharma Co., Ltd, Tokyo, Japan) were given intramuscularly. Inhalation anesthesia was used during the operation with a combination of isoflurane, dinitrogen monoxide, and oxygen. A chondral defect measuring 6 mm in diameter and 5 mm deep was made in the area of the host animal's medial femoral condyle using a biopsy punch and the damaged cartilage was covered with a three-layered chondrocyte sheet, which was stabilized with a nylon suture until initial fixation was achieved. This was performed in the left knees of 12 minipigs (aged 7–8 months and weighing 21.5–25.0 kg) in the transplantation group. At the same time, the articular cartilage of the medial femoral condyle was holed similarly in the right knees of 12 minipigs in the control group, but not covered with a cell sheet. Cartilage was harvested after 3 weeks, fixed in 4% paraformaldehyde (PFA) for 1 week and decalcified with K-CX decalcifying solution (Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan) for 1 week. Specimens were then embedded in paraffin wax, sectioned and stained with safranin-O for evaluation. Histological scoring of these sections was carried out by two observers, using the International Cartilage Repair Society (ICRS) grading system and the ICRS remodeling system to evaluate subchondral bone maintenance (Table 1).

2.5. Statistical analysis

The Mann–Whitney nonparametric U test was used to analyze histological scores in assessing the efficacy of treatment. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Chondrocyte sheets

Multilayered chondrocyte sheets were prepared by superimposing sheets and then culturing them together. It was thus possible to continue culturing three-layered sheets. As shown in Fig. 1, the layered articular chondrocyte sheets cultured for 3 weeks maintained their original shape without shrinking when the polyvinylidene difluoride (PVDF) membrane was removed. As a result, it was possible to create round grafts that demonstrated a chondrocytic phenotype.

3.2. Measurement of chondrocyte proliferation activity

Cell proliferation activities examined by MTT assay on days 3, 5 and 7 are illustrated in Fig. 2. Proliferation increased gradually until day 7; the numbers of chondrocytes increased by approximately 2.4-fold by day 5 and by approximately 6-fold by day 7.

3.3. Gross findings in the repaired cartilage

No distinct evidence of infection, articular damage at uninvolved sites, or synovial proliferation was observed in the transplantation or control groups. All defects were filled with white cartilaginous tissue, but defect filling in the control group was insufficient. The surface layer in the transplantation group had been replaced with smooth cartilaginous tissue of a color resembling that of healthy cartilage, but the surface layer in the control group had been replaced with coarse cartilaginous tissue, while the subchondral bone was exposed in some locations (Fig. 3a–d).

3.4. Histological findings of repaired cartilage

Good safranin-O staining and integration with surrounding tissue was noted in the transplantation group, which achieved sufficient cartilaginous repair and regeneration. All animals in the control group exhibited poor safranin-O staining, and tissue repair and regeneration were insufficient (Fig. 4a, b). ICRS scores were compared between the two groups. The mean score in the transplantation group was significantly higher than in the control group

Author's personal copy

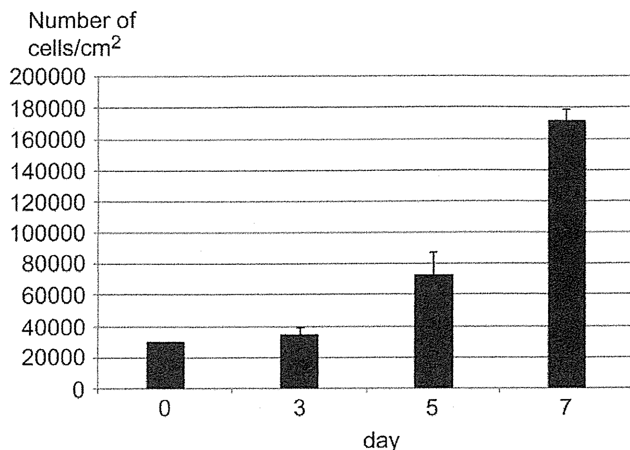


Fig. 2. Cell proliferation activity was examined by MTT assay. Cell proliferation increased steadily until day 7; the numbers of chondrocytes had increased approximately 2.4-fold by day 5 and approximately 6-fold by day 7.

(38.3 points versus 26.3 points, respectively, $p < 0.05$). Mean remodeling scores, an indication of subchondral bone condition, were significantly higher in the transplantation group (3.2 points) than in the control group (2.4 points; Fig. 5a, b). The ICRS scores in the transplantation and control groups are shown in Table 2.

In three of the 12 animals, a more detailed histological examination in the transplantation group revealed that, although tissue filling was present, safranin-O staining was poor and the subchondral bone in these animals was poorly repaired and regenerated (Fig. 4d). A comparison of ICRS scores indicated that subchondral bone repair was satisfactory (remodeling score 4 points) in the animals in the transplantation group that achieved adequate cartilage repair (histological score 42 points). However, subchondral bone repair was poor (remodeling score 2 points) in animals in the transplantation group with insufficient cartilage repair (histological score 29 points). These findings indicate that the degree of subchondral bone repair reflected that of cartilaginous tissue repair.

4. Discussion

Unless treated, full-thickness defects in knee cartilage cause secondary osteoarthritis and the resulting pain and poor joint function impact substantially on the activities of daily life. Recent advances in tissue engineering have prompted research on techniques to repair articular cartilage damage using a variety of transplanted cells. ACI was the first such technique investigated and is already used clinically in Western countries. However, the technique carries problems such as the potential for leakage of chondrocytes (implanted in suspension) outside the transplantation site, nonuniform distribution of transplanted cells [24] and damage to donor site tissues. Some have claimed that clinical outcomes are not superior to microfracture [25]. More effective treatments must therefore be developed. Ochi et al. [5], hypothesizing that the transplantation of 3D cartilaginous tissues composed of chondrocytes and matrix would yield better outcomes than chondrocyte transplantation, developed and clinically applied the transplantation of cultured chondrocytes embedded in atelocollagen gel. Furthermore, realizing the potential for bone marrow mesenchymal cells to differentiate, Wakitani et al. [26] developed and applied the transplantation of bone marrow mesenchymal cells embedded in collagen gel clinically. However, both types of implant were composites made of periosteum, a scaffold, bone marrow cells, cultured chondrocytes and numerous other elements and were thus unsuitable for creating an optimal environment for articular cartilage regeneration.

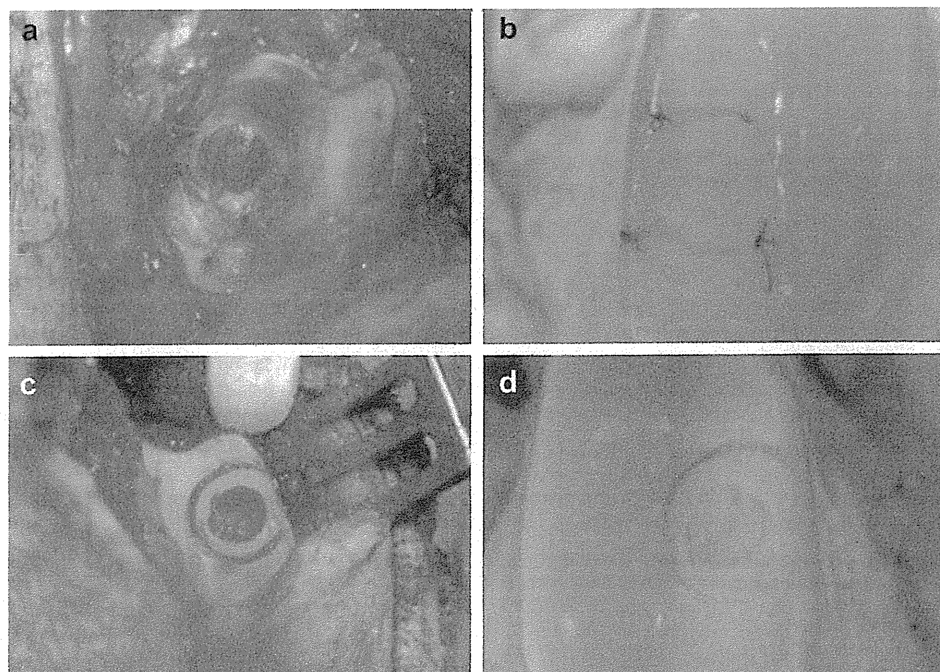


Fig. 3. Gross appearance (a, b, c, d) in the group receiving layered chondrocyte sheets (a, b) and the control group (c, d). Gross appearance is shown at transplantation (a, c) and 3 months later (b, d). The defect in the transplantation group (b) was filled with cartilaginous tissue, but filling in the defect of the control group (d) was insufficient and the subchondral bone was exposed partially. The defect of 6 mm in diameter and 5 mm deep was made, and outside circle of the defect is 8 mm in diameter, and is used for suturing chondrocyte sheet.

Author's personal copy

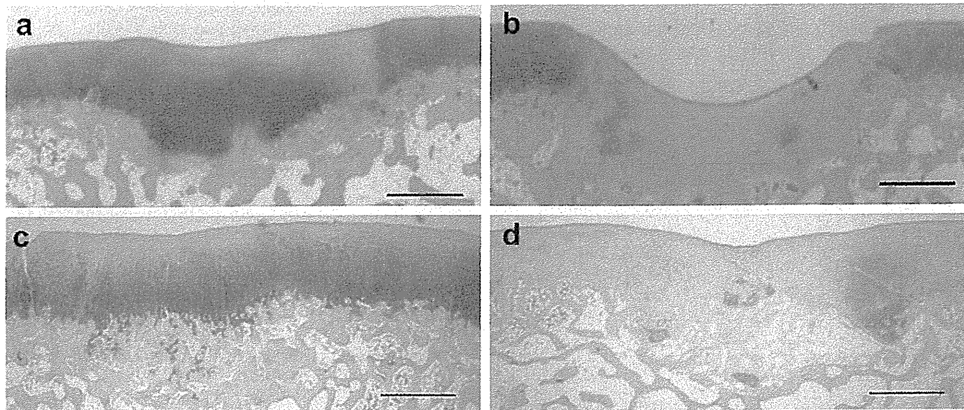


Fig. 4. Histology in the transplantation (a, c, d) and control groups (b). Safranin-O staining was robust and integration with the surrounding tissue was good in the transplantation group (a), but the control group (b) showed poor safranin-O staining and did not achieve satisfactory regeneration or repair. In (c), safranin-O staining was robust and subchondral bone repair and regeneration were good (International Cartilage Repair Society, ICRS histological grading system 42 points; remodeling score 4 points). Although the defect in (d) had been filled with tissue, safranin-O staining was poor and the subchondral bone was insufficiently repaired (ICRS histological grading system 29 points; remodeling score 2 points; scale bar = 2.0 mm).

Convinced that establishing an environment suited to tissue repair is essential for proper articular cartilage regeneration, we began basic research on repair and regeneration using only cells from bone marrow and cultured chondrocytes, without a scaffold or periosteum. Analysis of our layered chondrocyte sheets has shown that the chondrocytes maintain their phenotype, expressing aggrecan, collagen type II (COL2), SOX9 and COL27. The cells also express the adhesion molecules integrin α10 and fibronectin. Immunostaining confirmed the presence of COL2, integrin α10 and fibronectin in the cellular sheets [12,27]. Our findings indicate that these layered chondrocyte sheets demonstrate good adhesiveness and barrier functionality while maintaining a normal phenotype.

We also reported achieving robust tissue repair when covering only the surface layer with a layered chondrocyte sheet in research on partial-thickness defects in the articular cartilage of domestic rabbits [10]. This suggests that liquid factors from the layered sheets, in addition to the basic functionality of the sheets, contribute to repair.

In this study using chondrocytes from minipigs, which are large animals, we found that cultured chondrocytes had a high cell proliferation potential and that—as shown in previous studies—layered chondrocyte sheets created from these chondrocytes also contributed to the repair and regeneration of articular cartilage in this model of full-thickness defects. However, tissue having poor safranin-O staining, which was not noted in our domestic rabbit experiments, was identified in some of the animals (3/12) and the subchondral bone was poorly repaired in these animals. Vasara et al. [28] transplanted chondrocytes into a goat model of full-thickness defects. In animals with poor subchondral bone repair, the transplanted cells showed poor safranin-O staining, and integration with the surrounding tissue was insufficient. Muehleman et al. [29] transplanted chondrocytes into a minipig model of full-thickness defects and compared a group treated with risedronate to an untreated group using histology. Safranin-O staining of the transplanted cells was superior in the treated group and integration with the surrounding tissues was satisfactory,

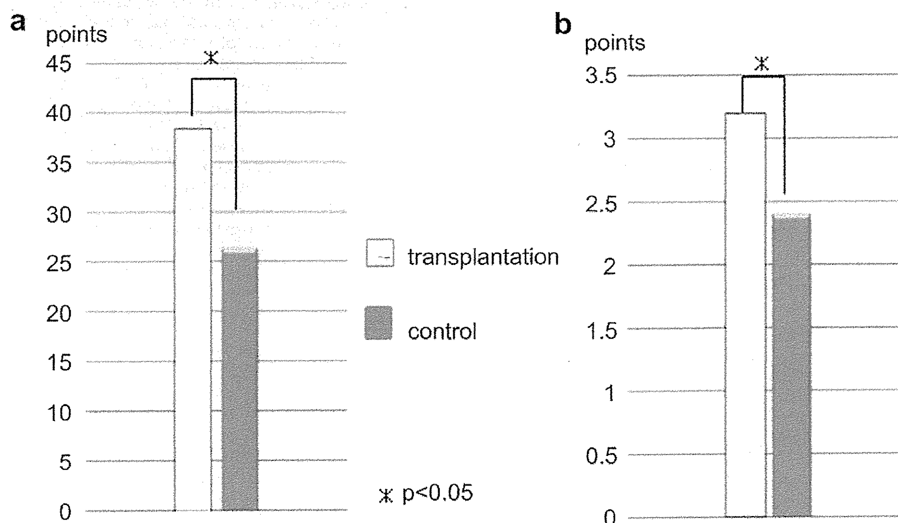


Fig. 5. ICRS histological grading system scores (a) and remodeling scores (b) in the transplantation and control groups. The mean histological score (a) in the transplantation group (38.3 points) was significantly higher than in the control group (26.3 points). Mean remodeling scores (b), an indication of subchondral bone condition, were significantly higher in the transplantation group than in the control group (3.2 points and 2.4 points, respectively).

Author's personal copy

Table 2
Results of ICRS histological grading system (Hgtot) and remodeling scores (Remod).

Case	Weight (kg)	Age (month)	Transplantation		Control	
			Hgtot	Remod	Hgtot	Remod
			(Points)		(Points)	
1	21.6	7	42	4	28	3
2	23.5	7	40	4	22	2
3	24.2	7	40	4	28	3
4	22.9	7	42	4	30	3
5	24.1	8	41	4	26	2
6	24.0	8	40	3	31	3
7	23.0	8	39	3	24	2
8	25.0	8	39	3	24	2
9	24.0	8	39	3	24	2
10	23.0	8	33	2	33	3
11	23.8	8	29	2	23	2
12	21.5	8	35	2	22	2
Average	23.4	7.7	38.3	3.2	26.2	2.4

These findings indicate that the points awarded in the ICRS histological grading system reflected the remodeling scores.

but greater than expected bone resorption occurred in this large animal model. Similarly, animals with poor subchondral bone repair in the transplantation group in the present study exhibited poor safranin-O staining in the regeneration tissue and insufficient integration with surrounding tissue. Risedronate administration and other measures to suppress bone resorption might thus be necessary in large animals used in future research to determine optimal transplantation conditions.

5. Conclusion

The use of layered chondrocyte sheets facilitated the repair and regeneration of tissue in a minipig model of full-thickness cartilaginous defects in the knee joints. Good safranin-O staining and integration with surrounding tissue was noted in the transplantation group, which achieved sufficient cartilaginous repair and regeneration. Some animals in the group receiving the layered chondrocyte sheets exhibited poor safranin-O staining of the repaired and regenerated tissue in the subchondral bone. Transplantation conditions and other factors must therefore be further investigated.

Acknowledgments

This work was supported by High-Tech Research Center Project 2004 for Private Universities and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare of Japan. All animal experiments were approved and carried out following the Guidelines of Tokai University on Animal Use.

References

[1] Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994;331:889–95.
[2] Matsusue Y, Yamamuro T, Hama H. Arthroscopic multiple osteochondral transplantation to the chondral defect in the knee associated with anterior cruciate ligament disruption. Arthroscopy 1993;9:318–21.
[3] Steadman JR, Rodkey WG, Briggs KK, Rodrigo JJ. The microfracture technic in the management of complete cartilage defects in the knee joint [in German]. Orthopade 1999;28:26–32.
[4] Minas T, Peterson L. Advanced techniques in autologous chondrocyte transplantation. Clin Sports Med 1999;18:13–44 [v–vi].
[5] Ochi M, Uchio Y, Kawasaki K, Iwasa J. Transplantation of cartilage-like tissue made by tissue engineering in the treatment of cartilage defects of the knee. J Bone Jt Surg Br 2002;84:571–8.
[6] Peterson L, Brittberg M, Kiviranta I, Akerlund EL, Lindahl A. Autologous chondrocyte transplantation. Biomechanics and long-term durability. Am J Sports Med 2002;30:2–12.
[7] Henderson IJ, Tuy B, Connell D, Osakes B, Hettwer WH. Prospective clinical study of autologous chondrocyte implantation and correlation with MRI at three and 12 months. J Bone Jt Surg Br 2003;85:1060–6.
[8] Marlovits S, Striessnig G, Resinger CT, Aldrian SM, Vecsei V, Imhof H, et al. Definition of pertinent parameters for the evaluation of articular cartilage repair tissue with high-resolution magnetic resonance imaging. Eur J Radiol 2004;52:310–9.
[9] Gilbert JE. Current treatment options for the restoration of articular cartilage. Am J Knee Surg 1998;11:42–6.
[10] Kaneshiro N, Sato M, Ishihara M, Mitani G, Sakai H, Mochida J. Bioengineered chondrocyte sheets may be potentially useful for the treatment of partial thickness defects of articular cartilage. Biochem Biophys Res Commun 2006; 349:723–31.
[11] Sato M, Ishihara M, Furukawa K, Kaneshiro N, Nagai T, Mitani G, et al. Recent technological advancements related to articular cartilage regeneration. Med Biol Eng Comput 2008;46:735–43.
[12] Mitani G, Sato M, Lee JI, Kaneshiro N, Ishihara M, Ota N, et al. The properties of bioengineered chondrocyte sheets for cartilage regeneration. BMC Biotechnol 2009;6:9–17.
[13] Shimizu T, Yamato M, Kikuchi A, Okano T. Two-dimensional manipulation of cardiac myocyte sheets utilizing temperature-responsive culture dishes augments the pulsatile amplitude. Tissue Eng 2001;7:141–51.
[14] Shimizu T, Yamato M, Isoi Y, Akutsu T, Setomaru T, Abe K, et al. Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. Circ Res 2002;90:40–8.
[15] Hirose M, Kwon OH, Yamato M, Kikuchi A, Okano T. Creation of designed shape cell sheets that are noninvasively harvested and moved onto another surface. Biomacromolecules 2000;1:377–81.
[16] Nishida K, Yamato M, Hayashida Y, Watanabe K, Yamamoto K, Adachi E, et al. Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. N Engl J Med 2004;351:1187–96.
[17] Harimoto M, Yamato M, Hirose M, Takahashi C, Isoi Y, Kikuchi A, et al. Novel approach for achieving double-layered cell sheets co-culture: overlaying endothelial cell sheets onto monolayer hepatocytes utilizing temperature-responsive culture dishes. J Biomed Mater Res 2002;62:464–70.
[18] Kushida A, Yamato M, Konno C, Kikuchi A, Sakurai Y, Okano T. Temperature-responsive culture dishes allow nonenzymatic harvest of differentiated Madin–Darby canine kidney (MDCK) cell sheets. J Biomed Mater Res 2000;51:216–23.
[19] Okano T, Yamada N, Okuhara M, Sakai H, Sakurai Y. Mechanism of cell detachment from temperature-modulated, hydrophilic–hydrophobic polymer surfaces. Biomaterials 1995;16:297–303.
[20] Kikuchi A, Okuhara M, Karikusa F, Sakurai Y, Okano T. Two-dimensional manipulation of confluently cultured vascular endothelial cells using temperature-responsive poly (*N*-isopropylacrylamide)-grafted surfaces. J Biomater Sci Polym Ed 1998;9:1331–48.
[21] Okano T, Yamada N, Sakai H, Sakurai Y. A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly (*N*-isopropylacrylamide). J Biomed Mater Res 1993;27:1243–51.
[22] Sekiya S, Shimizu T, Yamato M, Kikuchi A, Okano T. Bioengineered cardiac cell sheet grafts have intrinsic angiogenic potential. Biochem Biophys Res Commun 2006;341:573–82.
[23] Sato M, Asazuma T, Ishihara M, Kikuchi T, Masuoka K, Ichimura S, et al. An atelocollagen honeycomb-shaped scaffold with a membrane seal (ACHMS-scaffold) for the culture of annulus fibrosus cells from an intervertebral disc. J Biomed Mater Res A 2003;64:248–56.
[24] Sohn DH, Lottman LM, Kim SG, Pedowitz RA, Coutts RD, Sah RL. Effect of gravity on localization of chondrocytes implanted in cartilage defects. Clin Orthop Relat Res 2002;394:254–62.
[25] Knutsen G, Engebretsen L, Ludvigsen TC, Drogset JO, Grøntvedt T, Solheim E, et al. Autologous chondrocyte implantation compared with microfracture in the knee. A randomized trial. J Bone Jt Surg Am 2004;86A:455–64.
[26] Wakitani S, Imoto K, Yamamoto T, Saito M, Murata N, Yoneda M. Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees. Osteoarthritis Cartilage 2002;10:199–206.
[27] Kaneshiro N, Sato M, Ishihara M, Mitani G, Sakai H, Kikuchi T, et al. Cultured articular chondrocyte sheets for partial thickness cartilage defects utilizing temperature-responsive culture dishes. Eur Cell Mater 2007;13:87–92.
[28] Vasara AI, Hyttinen MM, Lammi MJ, Lammi PE, Langsjo TK, Lindahl AI, et al. Subchondral bone reaction associated with chondral defect and attempted cartilage repair in goats. Calcif Tissue Int 2004;74:107–14.
[29] Muehleman C, Li J, Abe Y, Pfister B, Sah RL, Philipps R, et al. Effect of risedronate in a minipig cartilage defect model with allograft. J Orthop Res 2009;27:360–5.
[30] Brehm W, Aklin B, Yamashita T, Rieser F, Trub T, Jakob RP, et al. Repair of superficial osteochondral defects with an autologous scaffold-free cartilage construct in a caprine model: implantation method and short-term results. Osteoarthritis Cartilage 2006;14:1214–26.
[31] O'Driscoll SW, Keeley FW, Salter RB. Durability of regenerated articular cartilage produced by free autogenous periosteal grafts in major full-thickness defects in joint surfaces under the influence of continuous passive motion: a follow-up report at one year. J Bone Jt Surg Am 1998;70:595–606.

軟骨再生

ラット膝関節内へ移植した細胞シートのBioluminescenceによる経時的評価

高久　裕子[*1)]　村井　邦彦[*1)]　鵜養　拓[*2)]
伊藤　聡[*2)]　小久保舞美[*2)]　小林　英司[*3)]
竹内　護[*1)]　持田　讓治[*2)]　佐藤　正人[*2)]

In vivo Cell Tracking by Bioluminescence Imaging after Transplantation of Bioengineered Cell Sheets to the Knee Joint

Yuko TAKAKU[*1)], Kunihiko MURAI[*1)], Taku UKAI[*2)], Satoshi ITO[*2)], Mami KOKUBO[*2)], Eiji KOBAYASHI[*3)], Mamoru TAKEUCHI[*1)], Joji MOCHIDA[*2)], Masato SATO[*2)]

臨整外 47：27～31，2012

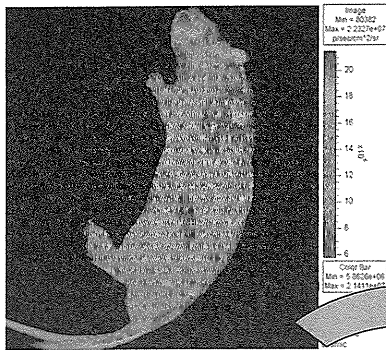
Key words：軟骨再生(cartilage regeneration)，生体発光イメージング(bioluminescence imaging)，細胞シート(cell sheet)

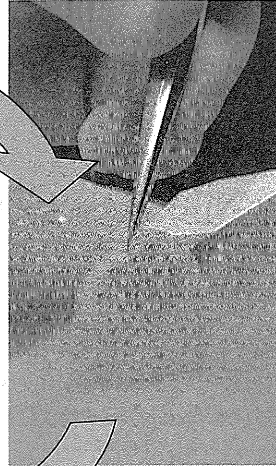
軟骨細胞シートは温度応答性培養皿を用いて酵素処理を必要とせずシート状に回収され，損傷した軟骨組織に対して優れた接着能をもち自己の軟骨修復能力を高める．すでに家兎およびミニブタを用いた動物実験において，膝関節症モデルへの同種細胞シート移植後の軟骨再生効果が実証されている．今回，細胞シートのラットにおける同種膝関節移植後の細胞動態について bioluminescence imaging (BLI)の手法を用いて検証したところ，関節内の長期生存を認め，移植細胞自身による軟骨再生効果が期待される結果となった．

はじめに

細胞シートは温度応答性培養皿を用いて酵素処理を必要とせずシート状に回収される．膝軟骨細胞は膝関節内を裏打ちする滑膜細胞が分泌する滑液(関節液)によって栄養されている．細胞シート作製時にこれらの細胞を共培養することで，軟骨細胞シートの作製期間の短縮が可能である．この組織工学的に構築された細胞シートによる関節軟骨の治療を目指している．いままでに家兎およびミニブタを用いた動物実験において，膝関節症モデルへの同種細胞シート移植後の軟骨再生効果が実証されている[6,8)]．近年，生物発光(bioluminescence) imaging (BLI)が移植細胞の *in vivo* 追跡に用いられているが，中でもホタルの発光遺伝子ルシフェラーゼ(*luc*)が最も頻用されている．発光基質ルシフェリンとの反応により放出される光量子を高感度 charge-coupled device (CCD)カメラで捕捉しイメージ化することで，実験動物を殺

[*1)] 自治医科大学医学部麻酔科学・集中治療医学講座〔〒329-0498 栃木県下野市薬師寺 3311-1〕Department of Anesthesiology and Critical Care Medicine, Jichi Medical University
[*2)] 東海大学医学部外科学系整形外科学　Department of Orthopaedic Surgery, Surgical Science, Tokai University School of Medicine
[*3)] 自治医科大学先端医療技術開発センター先端治療開発部門　Center of Development of Advanced Medcal Technology, Jichi Medical University





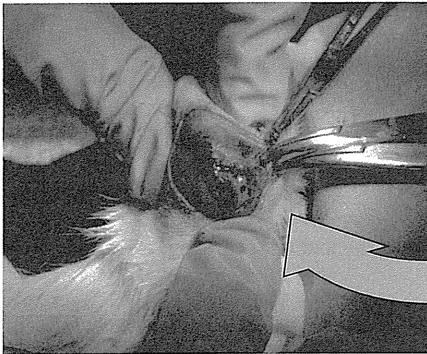


図1　Luc⁺ 軟骨細胞シートの移植
ルシフェラーゼを全身に発現するトランスジェニックラットの軟骨，滑膜組織から細胞シートを作製後，野生型 Lewis ラットの骨軟骨欠損部に移植した．

すことなく非侵襲的に同一個体を繰り返し経時的に長期追跡することが可能である．今回われわれはルシフェラーゼ遺伝子を導入したトランスジェニックラットより組織工学的に構築した細胞シートを用いて，ラット同種膝関節移植後の移植細胞動態を観察することができたので紹介する．

BLI

Bioluminescence とは，生体内で励起分子を作り出し，そのエネルギーにより発光する現象である．電気など他の化学発光に比べ発光量子効率に優れるため，熱を伴わない冷光と呼ばれ，発光スペクトルなども異なる．発光生物のなかでもホタルの発光量子効率が高く，発光基質ホタルルシフェリンの大量生産が可能になったことからバイオ研究のなかで最も頻用されている．発光基質ルシフェリンは発光酵素ルシフェラーゼと Mg^{2+} イオンを触媒として ATP と反応し発光する[3,5]．BLI とは発光する際に放出される光量子を高感度 CCD カメラで補足し画像イメージングする手法である．ルシフェラーゼ遺伝子を導入した遺伝子や細胞を用いて腫瘍細胞の観察，再生医学における移植細胞や免疫担当細胞の追跡，治療遺伝子の発現評価など，医学研究において近年飛躍的に広がりつつある[1,2,9]．

トランスジェニックラット

Kobayashi ら[4]は，ルシフェラーゼ遺伝子を発現するトランスジェニックラットを開発した（Rosa/Luciferase transgenic Lewis rats）．プロモーターに ROSA26 を有し，安定してルシフェラーゼを発現する．発光基質ルシフェリン投与すると，生体内のルシフェラーゼと ATP を使い自ら発光する．発光強度は細胞内 ATP や Mg^{2+} などの細胞環境に影響されるため，細胞種ごとに異なるが，同一種細胞においては投与したルシフェリン量に比例する．そのため，半定量的に生存細胞を追跡することも可能となる．ラット移植実験の細胞ソースとして有用である．

対象と方法

16週齢のRosa/Luciferase transgenic Lewis rats由来の軟骨細胞，滑膜細胞からLuc^+細胞シートを作製後，同週令の野生型Lewisラットの右膝大腿骨膝蓋面に18G針を用いて骨軟骨欠損を作製(ϕ；3 mm)，同部位にLuc^+細胞シートを同種移植した(図1)．群の作製としてLuc^+軟骨細胞(AC)シートとLuc^+滑膜細胞(SY)シートの2枚ずつの組み合わせによる群を作製した．すなわち，Luc^+軟骨細胞シート単独群(AC-AC group)とLuc^+滑膜細胞(SY-SY group)シート単独群，両シート併用群(AC-SY group)の3群を作製した(各n=4)．各種細胞シートの膝関節内滞在期間の影響について比較検討を試みた．D-luciferin(potassium salt；Biosynth) 150 mg/kgを肩甲骨下から皮下投与後，CCDカメラを搭載したIVISシステム(Xenogen Corp；Alameda，CA)(図2)を用いて繰り返し経時的にイメージングを行った．イメージングでは最も強いルシフェラーゼ発光強度を測定した．

結果

Luc^+移植細胞は1年以上イメージングにより追跡可能であった．ルシフェラーゼの発光シグナルは移植した右膝関節部位のみで検出され，他の部位では検出されなかった(図3)．

発光強度はAC-AC group，AC-SY group，SY-SY groupの順に強く測定された．この順序は移植後1カ月以降は変化しなかった．各群とも移植後3カ月頃から発光強度は安定し，明らかな増減を認めず，また1年の経過中にすべての個体イメージにおいて右膝のルシフェラーゼ発光は消失しなかった(図4)．

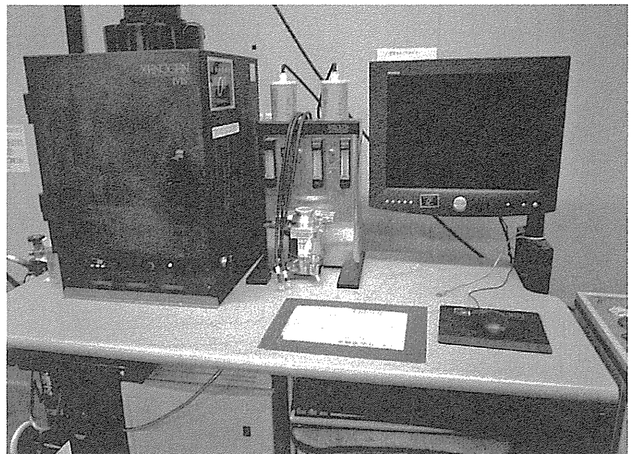


図2 IVISシステム(Xenogen社)
小動物体内におけるルシフェラーゼ遺伝子からの微弱な光を高感度CCDカメラで検出し，同時に被検体の普通写真を撮影した後，発光画像を重ね合わせてルシフェラーゼ発現の分布像を得る．

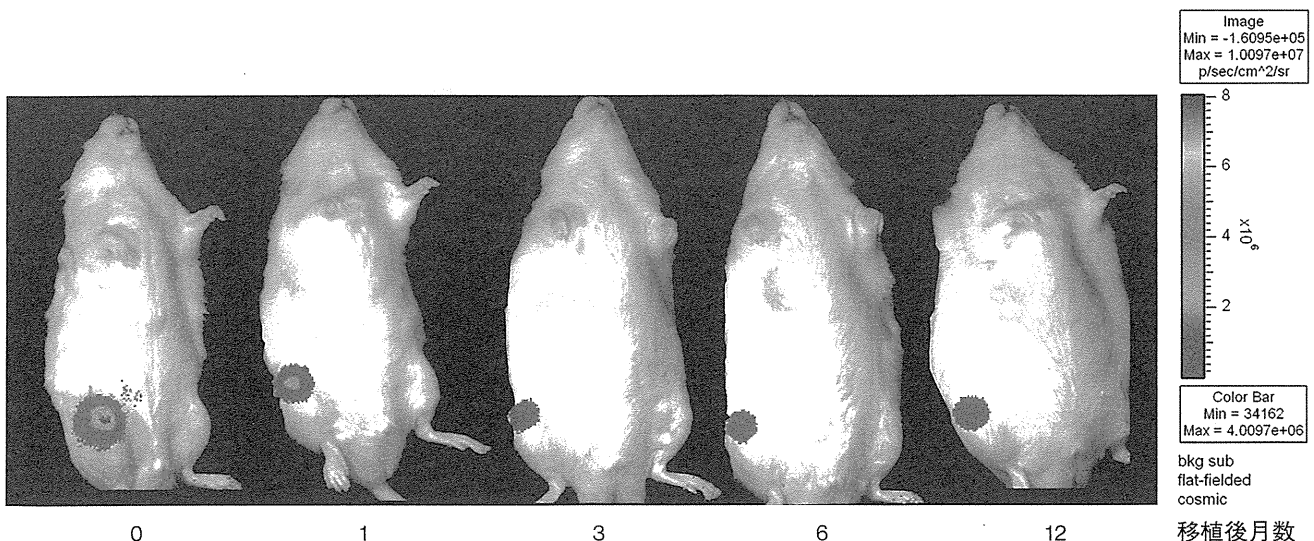


図3 AC-AC groupの細胞シート移植後の同一個体におけるCCDイメージ画像の継時的変化
カラー標識は発光強度を示す．ルシフェラーゼ発光を右膝部分にのみ認める．AC-AC group，AC-SY group，SY-SY groupの個体すべてに同様のイメージングが得られた．

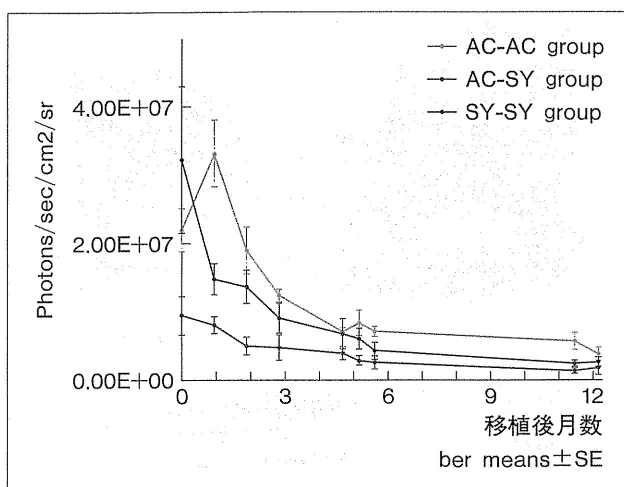


図4　細胞シート移植後の3群間の発光強度の推移
AC-AC group：Luc+ 軟骨細胞シート単独群
AC-SY group：Luc+ 軟骨細胞・Luc+ 滑膜細胞シート併用群
SY-SY group：Luc+ 滑膜細胞シート単独群

考察

BLIの手法を用いて長期に渡る細胞シートの関節内滞在期間を測定することに成功した．細胞シートはLuc+ 軟骨細胞シート，Luc+ 滑膜細胞シート，両細胞併用シートともに1年以上膝関節内に滞在し続けることをイメージングにより確認できた．イメージングでは右膝以外の発光シグナルを認めず，移植細胞は軟骨細胞，滑膜細胞ともに膝関節内にとどまって長期滞在し，異所への移動(転移)がないことが明らかになった．

細胞シート移植における軟骨の再生効果は，軟骨欠損部が細胞シートの優れた接着性によって覆われることで，関節液中の破壊因子から防御されるとともに，細胞シートからの成長因子を持続的に供給されることで，レシピエント自身の主導的な自己修復が促される[7,10]．そのため細胞シート由来の細胞は移植後2〜3週間で消失すると考えていたが，今回の結果はLuc+ 軟骨細胞シート由来の細胞自身が軟骨欠損部に生着して軟骨再生に寄与する可能性を明らかにした．細胞シートの発光強度は各群移植後，3カ月以降安定して経過するようになり，明らかな増減を認めなかった．ルシフェラーゼ発光強度は細胞数との関連があるが，軟骨細胞，滑膜細胞の細胞環境の差も影響していると考えられる．軟骨細胞と滑膜細胞を併用する効果は，3群とも長期生存を確認したため，1年間の滞在時間においては明らかではなかった．長期に観察されるルシフェラーゼ発光のシグナルはLuc+ 軟骨細胞，Luc+ 滑膜細胞シート由来の間葉系幹細胞の存在の関与も考慮され，今後の研究課題となった．

おわりに

本研究ではBLIの手法を用いて移植した細胞シートの軟骨細胞が関節内で長期滞在することを実証した．細胞シート自身が長期に残存し，軟骨再生に寄与する可能性が新しく示唆された．

謝辞

本研究は，平成21-23年度厚生労働科学研究費補助金再生医療実用化事業「細胞シートによる関節治療を目指した臨床研究」により実施されたものである．

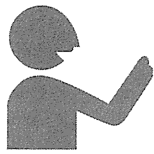
ここに謝意を表する．

文献

1) Contag CH, Ross BD : It's not just about anatomy : *in vivo* bioluminescence imaging as an eyepiece into biology. J Magn Rescon Imaging **16** : 378-87, 2002
2) Contag CH, Spilman S, Benaron D, et al : Visualizing gene expression in living mammals using a bioluminescent reporter. Photochem. Photobiol **66** : 523-531, 1997
3) Contag PR, Olomu IN, Stevenson DK, et al : Bioluminescent indicators in living mammals. Nat Med **4** : 245-247, 1998
4) Hakamata Y, Murakami T, Kobayashi E : "Firefly rats" as an organ/cellular source for long-term in vivo bioluminescent imaging. Transplantation **81** : 1179-1184, 2006
5) Hastings JW : Chemistries and colors of bioluminescent reactions : a review. Gene **173** : 5-11, 1996
6) Kaneshiro N, Sato M, Mochida J, et al : Bioengineered chondrocyte sheets may be potentially useful for the treatment of partial thickness defects of articular cartilage. Biochem Biophys Res Commun **349** : 723-731, 2006
7) Masuoka K, Asazuma T, Nemoto K, et al : Tissue engineering of articular cartilage using an allograft of cultured chondrocytes in a

membrane-sealed atelocollagen honeycomb-shaped scaffold (ACHMS scaffold). J Biomed Mater Res B Appl Biomater **75** : 177-184, 2005
8) Mitani G, Sato M, Mochida J, et al : The properties of bioengineered chondrocyte sheets for cartilage regeneration. BMC Biotechnol **9** : 17, 2009
9) Massoud TF, Gambhir SS : Molecular imaging in living subjects : seeing fundamental biological processes in a new light. Gens Dev **17** : 545-580, 2003
10) Nagai T, Sato M, Mochida J : Optimization of allograft implantation using scaffold-free chondrocyte plates. Tissue Eng Part A **7** : 1225-1235, 2008

リウマチ科，46(4)：406-414, 2011



話　題

抗VEGFヒト化モノクローナル抗体投与による関節軟骨修復*

長井敏洋**　佐藤正人**　持田讓治**

Key Words：cartilage repair, bevacizumab

はじめに

関節軟骨は硝子軟骨であり，疎な軟骨細胞と主にII型コラーゲンやプロテオグリカンなどの豊富な細胞外基質から構成される．その基質は含水性に富み約70～80％が水分である．そして血管を有しない関節軟骨は関節液からの拡散により栄養され，適度な粘弾性と非常に少ない摩擦係数を有し，円滑な関節運動が行われている．その関節軟骨の特徴的な構造つまり無血管組織であること，軟骨細胞周囲に密度の高い基質が存在していること，軟骨細胞が高度に分化しており分裂増殖しないことのために，いったん損傷が生じると通常の修復機転が働きにくいため，自己修復能力が著しく乏しい．そのため関節軟骨に損傷や変性が生じると徐々に変形性関節症へと進行する．

変形性関節症をはじめとする運動器疾患は，生命を直接脅かすものではないために，癌や心臓疾患など生命に直接かかわる疾患と比べるとやや軽視されているが，日常生活動作(ADL)を下げるばかりか，生活の質(QOL)の低下も招き，人的社会的損失は計り知れないものがある．2009年度版高齢社会白書によると，わが国の65歳以上の高齢者人口の総人口に占める割合(高齢化率)も22.7％となり，5人に1人が高齢者，10人に1人が75歳以上の人口という未曾有の超高齢社会が到来している．そして健康寿命を縮める原因(要支援となる原因)の第一位が関節疾患20.2％(2007年国民生活基礎調査)となっている．しかしながら，人工関節置換術などの手術適応のある末期の変形性関節症患者を除く初期から中期の患者に対しては漫然と保存的に加療されている現状がある．

関節軟骨修復法

従来から関節軟骨損傷に利用される骨髄刺激法に代表されるdrilling法[1)2)]，microfracture法[3)4)]は軟骨下骨まで欠損を作製し，骨髄から動員される軟骨前駆細胞(修復細胞)を損傷欠損部に供給することにより軟骨修復を期待する方法である．その修復過程は内軟骨性骨化による修復を機転としているため，欠損内部は修復細胞で満たされるが多くの血管侵入を認め，修復組織内部は骨に置換され，表層は主として線維軟骨で修復される[5)6)]．この方法は関節軟骨本来の硝子軟骨の修復でないにもかかわらず，非常に簡便な方法で侵襲も少なく安全性が確立されており，現在最も広く施行されている．しかしながら，線維軟骨による修復の是非に関しては論争中ではあるが，動物実験モデルにおいて線維軟骨による修復は長期経過において関節症性変化をひき起こすと報告されている[7)]．近年では骨軟骨移植法であるmosaic plasty法[8)]が行われるようになり良好な成績が報告され[9)]主流となってきている．この方法は自己の骨軟骨組織を関節非

* Repair of articular cartilage with anti-VEGF antibody bevacizumab.

** Toshihiro NAGAI, M.D., Ph.D., Masato SATO, M.D., Ph.D. & Joji MOCHIDA, M.D., Ph.D.: 東海大学医学部医学科外科学系整形外科学〔〒259-1193 伊勢原市下糟屋143〕; Department of Orthopaedic Surgery, Tokai University School of Medicine, Isehara 259-1193, JAPAN

図1 旋回培養法において作成したscaffold free軟骨プレート
直径は10mm.

荷重部から複数個採取し，骨軟骨欠損部にモザイク状に移植する方法である．ただし，採取できる骨軟骨片に限界があること，非荷重部ではあるが正常軟骨に欠損が残ること，移植部位の軟骨形状が再形成されないことなどの問題点が残る．

そこで再生医療による修復を期待してさまざまな治療法が開発・実施されている．黎明期からの細胞移植法[10)11)]そして近年では担体(scaffold)やバイオリアクターを用いた組織工学的手法において生体外で構築された軟骨様組織を移植する方法[12)13)]が考案され，1990年前半から海外で実施報告され，米国をはじめすでに2万例に近い手術症例の蓄積がある．しかしながら，細胞ならびに組織移植法は，健常部位からの組織採取の問題，移植片の安全性の問題，一定のクオリティーをもった移植片の供給など臨床応用への実現には高いハードルが存在する．また，実用化されても一般施設への普及にはcell processing center所有施設との間で採取組織や移植片の外部輸送が必要となり，その管理体制においても課題が多い．そしてさまざまな担体やバイオリアクターの開発，また担体を使用しない再生組織の構築などを含め，複雑化した製造工程においても真の関節軟骨を作ることが，想像以上に難しいことが明らかになっている．また，その対象疾患は小さな軟骨の外傷性病変にとどまり，再生医療が真に必要とされる変形性関節症の治療には20年近く経過した現在でも至っていない．

本稿ではこれまでに施行したわれわれの動物を用いた基礎的研究の紹介ならびに将来展望について述べる．

骨軟骨欠損モデルの基礎的研究

1. Scaffold free軟骨プレートを用いた動物実験モデル

以前の研究で，われわれは旋回培養法を用いてscaffoldを使用することなく3次元組織工学的軟骨を構築することに成功した[14)15)](図1)．日本白色家兎を用いた骨軟骨欠損モデルでの移植動物実験で欠損内部に骨髄から動員される軟骨前駆細胞(自己修復細胞)を満たし，軟骨分化への環境改変誘導因子として表層部のみにscaffold free組織工学的軟骨を移植し良好な長期間の修復効果を得た(図2)．その詳細な修復機序を検討したところ，移植群では移植後早期の骨髄由来の修復細胞で血管新生抑制因子であるコンドロモジュリン-I(ChM-I)の発現を認め，血管新生因子であるvascular endothelial growth factor(VEGF)の発現をほとんど認めなかった．一方，欠損放置群の修復細胞はChM-Iの発現を認めず，VEGFの強発現を認めた(図3)．つまり関節軟骨の特性であるanti-angiogenesis propertyを移植後早期に獲得したことで良好な修復再生効果を発揮することを確認した[16)]．

以上の研究により，骨軟骨欠損部に動員される修復細胞のVEGFの生物活性を一時的に阻止することで，細胞移植や組織移植を用いなくとも組織修復の環境改変効果が期待でき，小さな軟骨外傷のみならず変形性関節症の治療にまで踏み込め，移植療法に替わる軟骨再生医療として画期的なものであると仮説を立てた．

2. Bevacizumab(Avastin®)の静脈内投与による動物実験モデル

そこでわれわれはすでに医薬品として使用されている血管新生阻害剤である抗VEGFヒト化モノクローナル抗体bevacizumab(Avastin®)の静脈内投与による関節軟骨全層欠損に対する組織修復の環境改変効果を検討した[17)]．

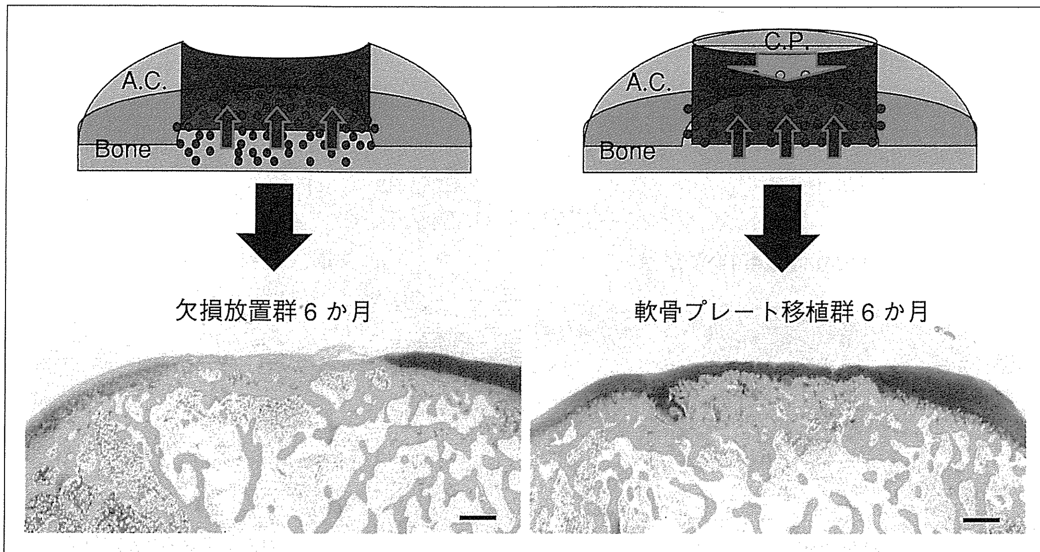


図 2　Scaffold free軟骨プレート移植の組織標本（サフラニン O）
上段は骨軟骨欠損モデルにおける欠損放置群と軟骨プレート移植群の修復過程のシェーマ．下段は術後 6 か月経過した組織標本．染色はサフラニン O 染色であり，barは 1 mm.

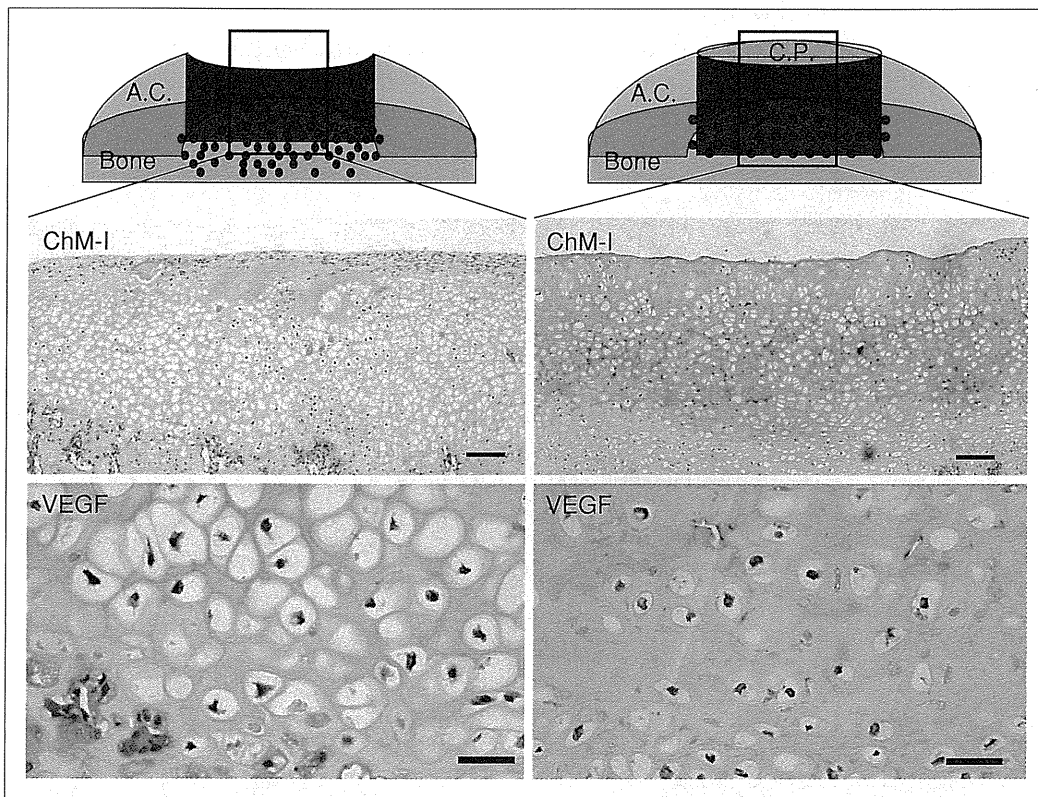


図 3　Scaffold free軟骨プレート移植の組織標本（ChM-I，VEGF）
上段は骨軟骨欠損モデルにおける欠損放置群と軟骨プレート移植群の修復過程のシェーマ．中段・下段ともに術後 1 か月の組織標本．中段はChM-Iの免疫染色でbarは200um．下段はVEGFの免疫染色でbarは50um.

本研究では12週齢の成熟日本白色家兎を使用した．傍膝蓋骨アプローチにおいて関節切開し大腿膝蓋関節に自然修復しない直径 5 mm深さ 3 mmの骨軟骨欠損を作製した．Bevacizumab投与群と欠損放置群を作製し，bevacizumab投与群では手術時と術後2週後に1羽につき100mgのbevacizumab

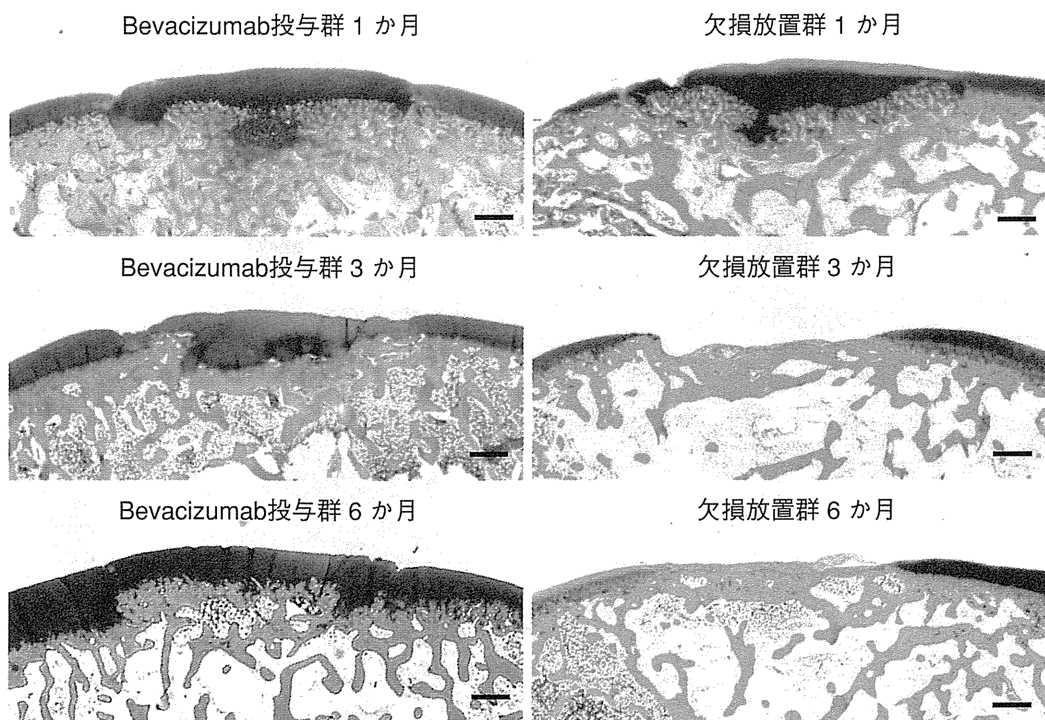


図４　骨軟骨欠損モデルにおけるbevacizumab投与効果
左側は骨軟骨欠損モデルにおけるbevacizumab投与群で右側は欠損放置群である．上段・中段・下段は順に術後１・３・６か月のサフラニンＯ染色であり，barは１mm.

を静脈内投与した．投与量は家兎に対する抗体感受性から決定した．術後４週，12週，24週で犠牲死させ，修復組織の評価をした．

術後１か月の欠損放置群の欠損内部はsafranin Oの染色性を認めたが，欠損表層において線維軟骨組織で修復された．一方，bevacizumab投与群の欠損内部はsafranin Oで濃染し，軟骨細胞様に分化した修復細胞で満たされており周囲軟骨との良好なintegrationを認め，欠損部は凸形で修復された．術後３か月の欠損放置群は線維性組織での修復を認めた．一方，bevacizumab投与群では，safranin Oの染色性を維持した修復を認め，軟骨下骨の修復も良好であった．修復組織内部の細胞の形態は硝子軟骨様で柱状配列構造を認めていた．術後６か月の欠損放置群においても軟骨下骨の露出や線維性組織での修復をうけ，周囲軟骨のsafranin Oの染色性の低下を認めた．一方，bevacizumab投与群では，safranin Oの染色性を維持した修復を認め，平滑な表層を保っていた(図４)．そこで，術後６か月のbevacizumab投与群の修復組織の免疫組織化学的評価をしたところ，硝子軟骨で認められるII型コラーゲンの発現を認め，I型コラーゲンの発現は認めなかった(図５)．また，関節軟

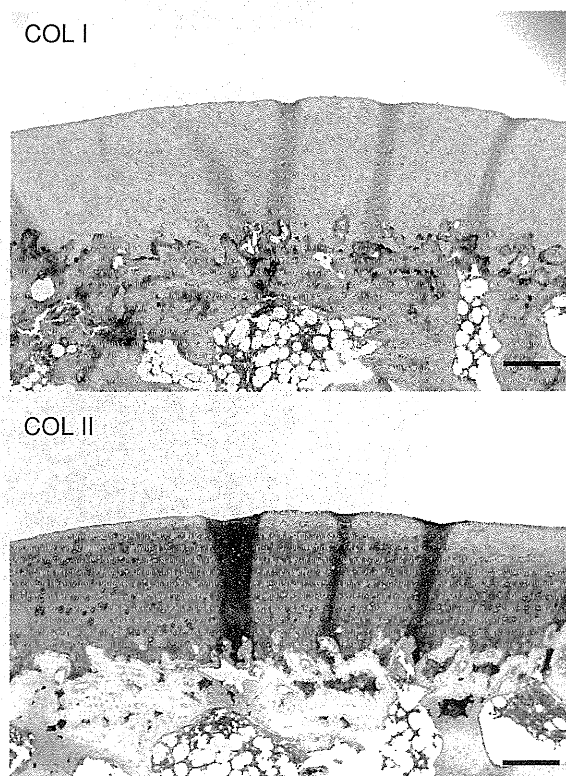


図５　骨軟骨欠損モデルにおけるbevacizumab投与群における術後６か月の組織標本
上段はコラーゲンタイプI・下段はコラーゲンタイプIIの免疫染色でbarは250umである．

骨の潤滑性に関与すると示唆されるsuperficial zone protein(SZP/PRG4)の発現を評価したところ，術後6か月のbevacizumab投与群における修復表層においてもSZPの発現を認めた(図6)．そして軟骨修復効果をInternational Cartilage Repair Society(ICRS)の評価基準(表1)を用いて検討したところ，術後1,3,6か月で欠損放置群と比較してbevacizumab投与群で有意に($P<0.01$)高値を示した(図7)．

欠損放置群とbevacizumab投与群の以上の結果の差違を検討するために，術後1か月における修復細胞の評価をした．欠損放置群ではChM-Iの

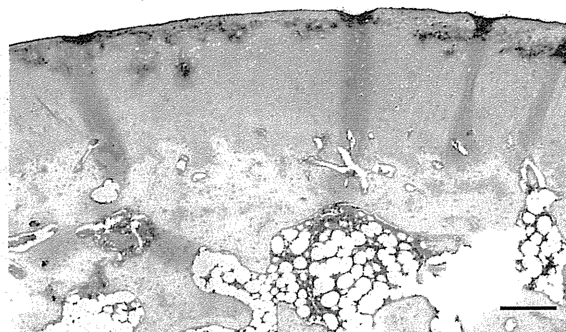


図6　骨軟骨欠損モデルにおけるbevacizumab投与群における術後6か月の組織標本
SZPの免疫染色でbarは250umである．

表1　International Cartilage Repair Society(ICRS)の組織評価基準

Variable	Comment
Ti	Tissue morphology 4=mostly hyaline cartilage 3=mostly fibrocartilage 2=mostly non-cartilage 1=exclusively non-cartilage
Matx	Matrix staining 1=none 2=slight 3=moderate 4=strong
Stru	Structural integrity 1=severe disintegration 2=cysts or disruption 3=no organization of chondrocytes 4=beginning of columnar organization of chondrocytes 5=normal, similar to healthy mature cartilage
Clus	Chondrocytes clustering in implant 1=25～100% of cells clustered 2=<25% of the cells clustered 3=no clusters
Tide	Intactness of calcified cartilage layer, formation of tidemark 1=<25% of the calcified cartilage layer intact 2=25～49% of the calcified cartilage layer intact 3=50～75% of the calcified cartilage layer intact 4=76～90% of the calcified cartilage layer intact 5=complete intactness of the calcified cartilage layer
Bform	Subchondral bone formation 1=no formation 2=slight 3=strong

Variable	Comment
SurfH	Histologic appraisal of surface architecture 1=severe fibrillation 2=moderate fibrillation 3=slight fibrillation or irregularity 4=normal
FilH	Histologic appraisal defect filling 1=<25% 2=26～50% 3=51～75% 4=76～90% 5=91～110%
Latl	Lateral integration of implanted material 1=not bonded 2=bonded at one end/partially both ends 3=bonded at both sides
Basl	Basal integration of implanted material 1=<50% 2=50～70% 3=70～90%
InfH	Inflammation 5=no inflammation 3=slight inflammation 1=strong inflammation
Hgtot	Histologic grading system Some of the histologic variables：tissue morphology(Ti), matrix staining(Matx), structural integrity (Stru), cluster formation(Clus), tidemark opening (Tide), bone formation(Bform), histologic surface architecture(SurfH), histologic degree of defect filling(FilH), lateral integration of defect-filling tissue (Latl), basal integration of defect-filling tissue (Basl), and histologic signs of inflammation(InfH) Maximum total：45 points

11～45 pointsでpointが高値であるほど良好な関節軟骨修復となる．

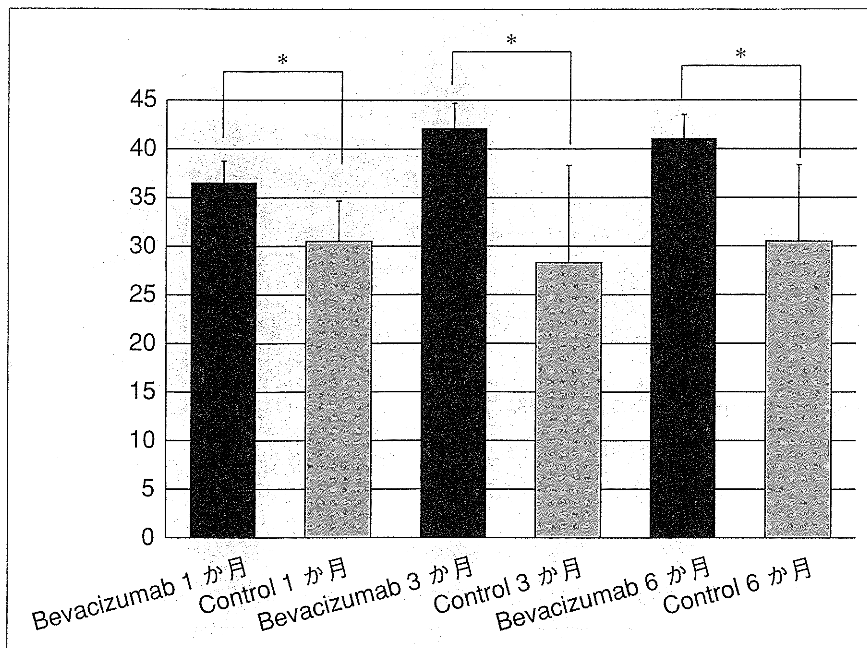


図7 ICRS組織評価
術後1・3・6か月におけるbevacizumab投与群と欠損放置群のICRSの組織評価であり，いずれにおいても有意差($P<0.01$)をもってbevacizumab投与群で高値である．

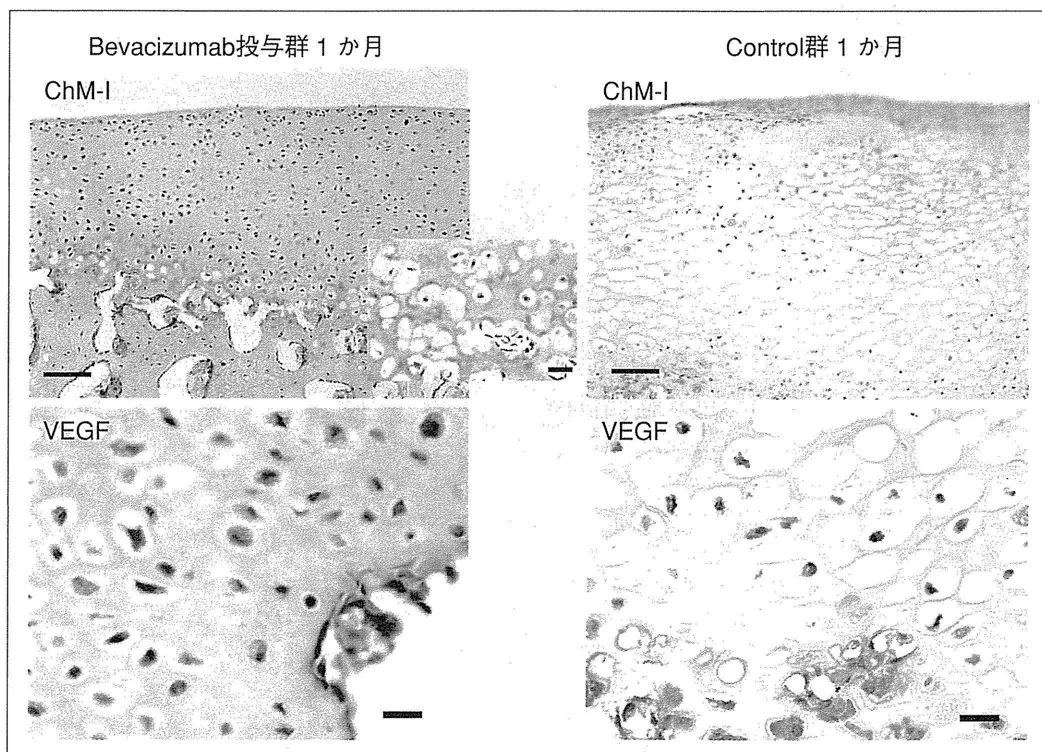


図8 骨軟骨欠損モデルにおけるbevacizumab投与効果—ChM-I，VEGFの免疫組織化学的染色による評価
左側は骨軟骨欠損モデルにおけるbevacizumab投与群で右側は欠損放置群である．上段・下段ともに術後1か月の組織標本．上段はChM-Iの免疫染色でbarは100um，強拡大は50um．下段はVEGFの免疫染色でbarは20um．

発現は認めなかったが，bevacizumab投与群ではChM-Iの発現を一様に認めた．VEGFにおいては，両群の修復細胞で一様に発現を認めた(図8).

VEGFは，多数の血管形成豊富な固形腫瘍や血液系悪性腫瘍で過剰発現する．したがって，VEGF経路を遮断することは，腫瘍学的な研究の主要焦点になった[18]．近年，悪性腫瘍治療薬として開発された抗VEGFヒト化モノクローナル抗体であるbevacizumabの治療効果が検討されている[19]．Bevacizumabは癌組織から分泌されるVEGFに結合することにより，血管内皮細胞に発現する受容体とVEGFとの結合を阻害し血管新生阻害をひき起こし，癌細胞の増殖を抑制すると報告されている[20)21]．また，bevacizumabは腫瘍のみならず血管新生の阻害治療の可能性が示唆されている[22]．

骨軟骨欠損の修復過程は，内軟骨性骨化による修復を機転としており，欠損内部は骨髄由来の修復細胞で満たされるが周囲からの血管侵入を認め，多くの修復組織の内部は骨に置換される[5)6]．また，肢芽発現の初期において病的な血管新生を介してVEGFは間葉組織の細胞凝集を妨げ[23]，軟骨形成の末期のVEGFの高発現は血管形成を通して内軟骨性骨化につながる[24]と報告されている．さらに，VEGFは関節炎をひき起こす最も重要因子の1つであることが示されており[25]，変形性関節症の軟骨細胞のVEGFの発現は軟骨破壊との関連が示唆されている[26]．また，われわれが以前に施行した組織工学的手法を用いた移植動物実験モデルでは，骨髄由来の修復細胞がanti-angiogenesis propertyを獲得したことで良好な軟骨修復再生効果を導くことを確認している[16]．そこでわれわれは，日本白色家兎を用いた骨軟骨欠損モデルに対して，抗VEGF抗体であるbevacizumabの静脈内投与による組織修復効果を試みた．その結果，術後6か月において良好な軟骨修復過程を確認した．興味深いことは，術後早期におけるbevacizumab投与群の修復組織でChM-Iの発現を獲得したことである．ChM-Iは，無血管組織として関節軟骨を維持し，軟骨損傷に対し再生を促進すると報告されている[27]．本研究でのChM-Iの発現は，軟骨下骨からの血管の侵入を阻止し，関節軟骨の形質発現を獲得するbarrierの働きをしていると考えられる．

しかしながら，VEGFは肢芽発現の際，幹細胞ならびに軟骨細胞のsurvival factorであるとも報告されている[28)29]．本研究では，抗VEGFヒト化モノクローナル抗体であるbevacizumabを骨軟骨欠損部に動員される修復細胞の軟骨分化へのinitiatorとして使用して一時的な使用に留めている．そのため，手術当日と術後2週後の術後早期の投与としており，術後の修復細胞のVEGFの生物活性を完全に阻止しているわけではない．実際，術後1か月の欠損内部で，bevacizumab投与群においてもVEGFの発現を認めていた．また，軟骨下骨からの血管侵入をうける修復細胞の層ではanti-angiogenesis factorであるChM-Iの発現を認めていた．つまり関節軟骨の良好な修復に重要なことは，修復細胞のangiogenesisとanti-angiogenesisのpropertyのbalance，つまり至適なVEGF signalの調整であることが示唆された．

本研究の手法は，関節鏡視下にdrilling法やmicrofracture法を施行し，術後にbevacizumabを静脈内投与するのみであり非常に簡便な方法である．また，既存の軟骨再生医療と併用も可能であるため，さらなる修復効果の向上が期待できる．

将来の展望

現在，われわれは家兎膝前十字靱帯切離モデルを軟骨部分損傷モデル(外傷後変形性関節症モデル)として使用しbevacizumabの投与効果を検討している．変形性関節症において常に混在しながら存在する軟骨部分損傷と骨軟骨損傷の両タイプの軟骨損傷に対してbevacizumab投与による治療効果を示していく予定である．つまり抗VEGFヒト化モノクローナル抗体の静脈内投与のみで，変形性関節症に対する関節軟骨の変性抑制効果を検討することであり，新規の変形性関節症に対する治療法の確立を目指すものである．本薬剤はすでに臨床で使用されており，適応の拡大により，広く多くの施設で治療施行可能となる見込みである．初期から中期の変形性関節症患者を早い時期に根治的に治療することが可能となり，生涯自分自身の関節で過ごせるよう