Figure 6. Evaluation of sulfated GAG. There was decrease in the amount of sulfated GAG in PGA(Sn)-treated cultured cells compared with that of the control. All experiments were run in quadruplicate for two separate times.

pared with that of the control (Fig. 6). However, in the same experiment, almost no difference in this amount was observed between the PGA-treated culture and the control.

DISCUSSION

Attempts to identify a perfectly biocompatible and biodegradable polymer have been ongoing over the past decade. An ideal biomaterial should fulfill its purpose satisfactorily and then biodegrade to obviate any risk of foreign body reaction.[31] Synthetic biodegradable polymers, especially those belonging to the polyester family, have played an important role in a number of tissue engineering efforts. PGA, an aliphatic polyester, can be degraded in two ways: by hydrolysis and by nonspecific esterases and carboxypeptidases, followed by either excretion in the urine or entrance into the tricarboxylic acid cycle.[32]

Several different catalysts, namely organotin, antimony, zinc, and lead, are used in the polymerization process to synthesize high molecular weight PGA. Different tin compounds were observed to produce general cytotoxic effects in rabbit articular cartilage in monolayer culture,[33] and bone is suggested to be the critical organ in inorganic tin toxicity in rats.[26] Therefore, in this study, we aspired to evaluate the chondrogenic effects of HAC with PGA synthesized with and without an inorganic tin catalyst, with the aim of clarifying the biocompatibility of inorganic tin as a catalyst for future clinical use.

It was reported that oral administration of certain tin compounds at specific concentrations exerted stimulatory effects on chondrocyte proliferation in the rat.[33] Consistent with this, the proliferation assay performed in our study also showed that HAC with PGA(Sn) had stimulatory effects on chondrocyte proliferation in micromass culture (Fig. 1). On the other hand, PGA neither stimulated nor inhibited the chondrocyte proliferation, and thus, inorganic tin as catalyst seemed to play a stimulatory role in HAC proliferation. In our experiment, PGA with inorganic tin as the catalyst caused almost no change in cell differentiation, but PGA-treated cultures did show a significant decrease when compared with that of the control (Fig. 2). Furthermore, quantitative estimation of extracellular matrix gene expression by real-time PCR confirmed that the cartilage-specific protein, collagen type II, was more strongly expressed in PGA(Sn)- than in PGA-treated cultured chondrocytes [Fig. 3(A)]. However, the expression of the aggrecan gene was inhibited in the PGA culture, but no difference was observed between the PGA(Sn) and the control cultures [Fig. 3(B)].

It was reported that oral administration of inorganic tin caused a decrease in the proliferation of chondrocytes, accompanied by suppression of DNA synthesis with subsequent inhibition in collagen synthesis in rat.[34] On the contrary, our results showed enhancement of proliferation, expression of the collagen type II gene, and amount of collagen type II protein by *in vitro* culture of HAC with PGA(Sn). We speculated that difference in the route of administration might be the cause of these diverse effects of inorganic tin compound. As mentioned earlier, monolayer culture of rabbit articular cartilage with tin compounds caused inhibition in the synthesis of core proteins, followed by a decrease in the synthesis of sulfated GAG.[33] In agreement with this result, our report also showed a decrease in the amount of sulfated GAG by culture of HAC with PGA(Sn). A study performed in our laboratory using HAC in a micromass culture system has already shown that PGA synthesized with organic tin catalyst caused a decrease in cell proliferation, but a significant increase in cell differentiation [29] and was completely contradictory to our present results. The molecular weight of PGA(Sn), and the type of tin product such as $SnCl_2$ and dibutyl tin were thought to be the key factor of different effects of chondrogenesis on HAC.

To the best of our knowledge, no other study has yet investigated the chondrogenic effects of PGA with inorganic tin as a catalyst, using HAC in a micromass culture system. This study is the first to show the biological action of inorganic tin as catalyst in PGA on human articular chondrogenesis in a micromass culture system. Our observation revealed that low concentration of inorganic tin when used in the polymer

of PGA showed enhancing effects of tin compounds on chondrocytes in comparison to without tin polymer because of increase in the permeability of inorganic tin under the presence of PGA. However, further study is required for the application of this PGA(Sn) in clinical practice.

References

1. Yasui N, Osawa S, Ochi T, Nakashima H, Ono K. Primary culture of chondrocytes embedded in collagen gels. Exp Cell Biol 1982;50:92–100.

2. Kimura T, Yasui N, Ohsawa S, Ono K. Chondrocytes embedded in collagen gels maintain cartilage phenotype during long-term cultures. Clin Orthop 1984;186:231–239.

3. Grandolfo M, D'Andrea P, Paoletti S, Martina M, Silvestrini G, Bonucci E, Vittur F. Culture and differentiation of chondrocytes entrapped in alginate gels. Calcif Tissue Int 1993;52:42–48.

4. Loty S, Sautier JM, Loty C, Boulekbache H, Kokubo T, Forest N. Cartilage formation by fetal rat chondrocytes cultured in alginate beads: a proposed model for investigating tissue-biomaterial interactions. J Biomed Mater Res 1998;42:213–222.

5. Perka C, Spitzer RS, Lindenhayn K, Sittinger M, Schultz O. Matrix-mixed culture: new methodology for chondrocyte culture and preparation of cartilage transplants. J Biomed Mater Res 2000;49:305–311.

6. Meinhart J, Fussenegger M, Hobling W. Stabilization of fibrin-chondrocyte constructs for cartilage reconstruction. Ann Plast Surg 1999;42:673–678.

7. Perka C, Schultz O, Lindenhayn K, Spitzer RS, Muschik M, Sittinger M, Burmester GR. Joint cartilage repair with transplantation of embryonic chondrocytes embedded in collagen-fibrin matrices. Clin Exp Rheumatol 2000;18:13–18.

8. Perka C, Schultz O, Spitzer RS, Lindenhayn K, Burmester GR, Sittinger M. Segmental bone repair by tissue-engineered periosteal cell transplants with bioresorbable fleece and fibrin scaffolds in rabbits. Biomaterials 2000;21:1145–1153.

9. George-Weinstein M, Gerhart JV, Foti GJ, Lash JW. Maturation of myogenic and chondrogenic cells in the preosmitic mesoderm of the chick embryo. Exp Cell Res 1994;211:263–274.

10. Ashammakhi N, Rokkanen P. Absorbable polyglycolide devices in trauma and bone surgery. Biomaterials 1997;18:3–9.

11. Middleton JC, Tipton JA. Synthetic biodegradable polymers as orthopedic devices. Biomaterials 2000;21:2335–2346.

12. Kohn J, Langer R. Bioresorbable and bioerodible materials. In: Ratner BD, Hoffman AS, Schoen FJ, Lemon JE, editors. An Introduction to Materials in Medicine. San Diego: Academic Press; 1997. p 65–73.

13. Wong WH, Mooney DJ. Synthesis and properties of biodegradable polymers used as synthetic matrices for tissue engineering. In: Atala A, Mooney D, editors. Synthetic Biodegradable Polymer Scaffolds. Boston: Burkhauser; 1997. p 51–84.

14. Yaszemsky MJ, Payne RG, Hayes WC, Langer R, Mikos AG. Evolution of bone transplantation: Molecular, cellular and tissue strategies to engineer human bone. Biomaterials 1996;17:175–185.

15. Burg KJL, Porter S, Kellam JF. Biomaterials development for bone tissue engineering. Biomaterials 2000;21:2347–2359.

16. Tessa H, Sundback C, Hunter D, Cheney M, Vacanti JP. A polymer foam conduit seeded with Schwann cells promoted guided peripheral nerve regeneration. Tissue Eng 2000;6:119–127.

17. Bryan DJ, Holway AH, Wang KK, Silva AE, Trantolo DJ, Wise D, Summerhayes IC. Influence of glial growth factor and Schwann cells in a bioresorbable guidance channel on peripheral nerve regeneration. Tissue Eng 2000;6:129–138.

18. Evans GRD, Brandt K, Widmer MS, Lu L, Meszlenyi RK, Gupta PK, Mikos AG, Hodges J, Williams J, Gurlek A, Nabawi A, Lohman R, Patrick JCR. In vivo evaluation of poly(L-lactic acid) porous conduits for peripheral nerve regeneration. Biomaterials 1999;20:1109–1115.

19. Matsumoto K, Ohnishi K, Sekine T, Ueda H, Yamamoto Y, Kiyotani T, Nakamura T, Endo K, Shimizu Y. Use of a newly developed artificial nerve conduit to assist peripheral nerve regeneration across a long gap in dogs. ASAIO J 2000;46:415–420.

20. Salanki Y, D'eri Y, Platokhin A, Sh-Rozsa K. The neurotoxicity of environmental pollutants: the effect of tin (Sn2+) on acetylcholine-induced currents in growing pond snail neurons. Neurosci Behav Physiol 2000;30:63–73.

21. Gyori J, Platoshyn O, Carpenter DO, Salanki J. Effect of inorganic and organic tin compounds on Ach- and voltage-activated Na currents. Cell Mol Neurobiol 2000;20:591–604.

22. Chang LW. The neurotoxicology and pathology of organomercury, organolead, and organotin. J Toxicol Sci 1990;15:125–151.

23. de Mattos JC, Dantas FJS, Bezerra RJAC, Bernardo-Filho M, Cabral-Neto JB, Lage C, Leitao AC, Caldeira-de-Araujo A. Damage induced by stannous chloride in plasmid DNA. Toxicol Lett 2000;116:159–163.

24. Chao JS, Wei LY, Huang MC, Liang SC, Chen HH. Genotoxic effects of triphenyltin acetate and triphenyltin hydroxide on mammalian cells in vitro and in vivo. Mutat Res 1999;21:167–174.

25. De Santiago A, Aguilar-Santelises M. Ogranotin compounds decrease in vitro survival, proliferation and differentiation of normal human B lymphocytes. Hum Exp Toxicol 1999;18:619–624.

26. Yamaguchi M, Kitade M, Okada S. The oral administration of stannous chloride to rats. Toxicol Lett 1980;5:275–278.

27. Chang LW. Hippocampal lesions induced by trimethyltin in neonatal rat brain. Neurotoxicology 1984;5:205–215.

28. Tsuchiya T, Ikarashi Y, Arai T, Ohhashi J, Nakamura A. Improved sensitivity and decreased sample size in a cytotoxicity test for biomaterials: a modified colony microassay using a microplate and crystal violet staining. J Appl Biomater 1994;5:361–367.

29. Rahman MS, Tsuchiya T. Enhancement of chondrogenic differentiation of human articular chondrocytes by biodegradable polymers. Tissue Eng 2001;7:781–790.

30. Brown AN, Kim BS, Alsberg E, Mooney DJ. Combining chondrocytes and smooth muscle cells to engineer hybrid soft tissue constructs. Tissue Eng 2000;6:297–305.

31. Gristina AG. Biomaterial-centered infection: microbial adhesion versus tissue integration. Science 1987;237:1588–1595.

32. William SDF. Some observations on the role of cellular enzymes in the in vivo degradation of polymers. In: Syrett BC, Acharya A, editors. Corrosion and degradation of implant materials 1979. p 61–75.

33. Webber RJ, Dollins SC, Harris M, Hough AJ Jr. Effect of alkyltins on rabbit articular and growth-plate chondrocytes in monolayer culture. J Toxicol Environ Health 1985;16:229–242.

34. Yamaguchi M, Sugii K, Okada S. Inhibition of collagen synthesis in the femur of rats orally administered stannous chloride. J Pharm Dyn 1982;5:388–393.

Improved Biocompatibility of Titanium–Zirconium (Ti–Zr) Alloy: Tissue Reaction and Sensitization to Ti–Zr Alloy Compared with Pure Ti and Zr in Rat Implantation Study

Yoshiaki Ikarashi[1], Kazuhiro Toyoda[2,*], Equo Kobayashi[3], Hisashi Doi[3],
Takayuki Yoneyama[3], Hitoshi Hamanaka[3] and Toshie Tsuchiya[1]

[1]*Division of Medical Devices, National Institute of Health Sciences, Tokyo 158-8501, Japan*
[2]*Division of Pathology, National Institute of Health Sciences, Tokyo 158-8501, Japan*
[3]*Department of Metallurgy, Division of Biomaterials, Institute of Biomaterials and Bioengineering,*
Tokyo Dental and Medical University, Tokyo 101-0062, Japan

Titanium–zirconium (Ti–Zr) binary alloy has better corrosion resistance and mechanical properties than commercially pure Ti. The present study was designed to determine the biocompatibility of Ti–Zr alloy by an implantation test in animal bodies in comparison with pure Ti, Zr, and chromium (Cr) implants as positive controls. Sample specimens were placed in a subcutaneous position in rats for 8 months. No significant decreases in body weight, the weight of any organ, or the weight of any organ relative to body weight were found in the implant groups compared to a no-implant control group. On hematological examination, small differences in several parameters were found in some groups, but these changes were not attributable to the materials implanted. Mitogen-induced blastogenesis was observed in similar degrees among all implant groups. These results suggest that the implantation of test samples did not cause systemic toxicity or a decrease in immune activity. The fibrous capsule membranes around the Ti and Ti–Zr alloy implants were thinner than those around Cr implants. The numbers of macrophages, inflammatory cells, and other cells involved in immune responses in and around the fibrous capsules of the Cr- and Ti-implant groups were higher than those of the Ti–Zr alloy- and Zr-implant groups. The Ti–Zr alloy had the lowest total score of tissue responses among the materials tested. None of the animals from the Ti-, Zr-, and Ti–Zr alloy-implant groups exhibited a skin reaction following exposure to Ti or Zr salt solutions. These results indicate the Ti–Zr alloy has better biocompatibility than Ti for use as an artificial surgical implant.

(Received April 28, 2005; Accepted August 31, 2005; Published October 15, 2005)

Keywords: titanium alloy, titanium, biocompatibility, inflammation, hypersensitivity

1. Introduction

Stainless steel and cobalt–chromium (Co–Cr) alloys have been widely used as materials in orthopedic and dental implants because of their biocompatibility, physical properties, and manufacturing ease.[1] In general, these metallic alloys have excellent corrosion resistance and are not believed to cause any local or systemic responses. However, fretting corrosion of metallic implants is sometimes observed in contact with biological systems, causing the release of metallic ions from the implants.[2,3] Elevated levels of metal ions have been found in blood, urine, and tissues of patients and animals that have received metal implants.[2–6] Metallic ions, such as nickel (Ni), Co, and Cr, are known to cause adverse tissue reactions and allergy.[7–13]

Titanium (Ti) and its alloys are currently considered the most attractive metallic materials for orthopedic and dental surgery. The use of Ti alloys is increasing due to their excellent mechanical strength, corrosion resistance, and good biocompatibility.[14–17] These properties are attributable mainly to the formation of a stable titanium oxide (TiO_2) layer on the surface.[18,19] However, the mechanical/tensile strength of commercially pure Ti is insufficient for its use as an artificial hip joint, pin, or screw,[20] and its wear resistance is also inferior to that of stainless steels and Co–Cr alloys.[20] The appearance of increased wear debris from Ti has been associated with inflammation, bone resorption, and pain.[18,19,21–24] To improve mechanical strength and wear resistance, various elements have been added to create new

Ti alloys. Ti–6Al–4V alloy is a high-strength Ti alloy, but its biocompatibility is considered lower than that of commercially pure Ti. The wear resistance and corrosion resistance of Ti–6Al–4V alloy are inferior to those of Ti, and Ti–6Al–4V alloy releases compounds and wear debris containing vanadium (V) or V ion, both of which are toxic.[18] At present, it is difficult to avoid the wear and/or fretting of implanted alloys in a living body, resulting in the release of elements contained in the alloy and the formation of wear debris. Therefore, it is preferable not to use highly toxic elements in alloys.

Zirconium (Zr) belongs to the VIa group in the periodic table, as does Ti, and is known to have chemical properties similar to those of Ti.[20] An insoluble oxide is formed on the surface in the air, and the surface oxide composition (zirconia) influences corrosion behavior. There is general agreement that Zr compounds have no local or systemic toxic effects.[25] Based on this apparent lack of toxicity, Kobayashi *et al.* selected Zr as an alloying element to improve the properties of commercially pure Ti, and prepared a Ti–Zr binary alloy as a material for use in medical devices, such as artificial joints or bone plates.[20] The hardness of Ti–50%(atom%) Zr alloy is 2.5 times as large as that of commercially pure Ti, suggesting the alloy's superior mechanical strength.[20]

Besides mechanical properties, the biocompatibility of an alloy is important if it is to be used in implant devices. We previously observed that animals were sensitized to Cr by long-term implantation of corrosive Cr alloys.[26] The animal model is a prevalent tool in examining tissue responses to implant material. The present study examined the biocom-

*Present address: Japan Tabacco Inc., Tokyo 130-8603, Japan

patibility of the Ti–Zr alloy compared with those of Ti and Zr by implantation tests using rats. Pure chromium (Cr) was used as a positive control material. After 8 months' implantation to a subcutaneous position, the reaction of the tissue around the specimen was studied hematologically and histologically. Since cases of sensitization to pacemakers which are made of Ti have been reported,[27,28] we topically applied a metal salt solution to rats in order to detect hypersensitivity to the metal. Further, the *in vitro* proliferation of spleen lymphocytes was also measured to evaluate immune system activity.

2. Materials and Methods

2.1 Test materials

Specimens of Ti, Zr, Ti–Zr binary alloy (Ti containing 50 atom% Zr), and Cr were used. The preparation of these specimens was reported in detail by Kobayashi *et al.*[20] Briefly, the specimens were prepared from sponge Ti (>99.8 mass% purity), Zr (>99.5 mass% purity), and electrolytic Cr metal (>99.98 mass% purity) by arc melting with a non-consumable tungsten electrode on a water-chilled copper hearth under an ultra-high-purity argon atmosphere. To prevent the macroscopic composition gradient by insufficient mixing, ingots were turned over and re-melted at least five times. To minimize mechanical trauma during implantation, the specimens were prepared in the form of plates with a diameter of 14 mm and a thickness of 1 mm, and were cut out from these button ingots using a silicon carbide wheel cutter. The plate specimens were polished mechanically to a mirror finish using emery paper (gradually finer, up to 600 grit) followed by 0.03 mm alumina paste. Each specimen was washed with 70% ethanol and ultra-pure water, and autoclaved.

2.2 Animals

Female F344/DuCrj rats, 5 weeks old, were obtained from Charles River Japan Inc. (Kanagawa, Japan) and randomized into five groups of six rats each. The animals were housed in air-conditioned facilities (temperature $23 \pm 2°C$, relative humidity $55 \pm 5\%$, light cycle 12 h/day). Diet (F-2, Funabashi Farm Co., Chiba, Japan) and water were available ad libitum throughout the experimental period. After a 1-week acclimation period, all animals were used in the implantation test. All animals were cared for according to the Japan animal rights act and the NIHS-Japan guidelines for the care and use of laboratory animals.

2.3 Implantation procedure[26,29]

The animals were anesthetized with 50 mg/kg Nembutal (sodium pentobarbital, Dainabot Co., Ltd., Osaka, Japan) *via* intraperitoneal injection. After the induction of anesthesia, the hair on the back was shaved around the implantation site and the skin was sterilized by brushing with a 70% ethanol solution. An incision was made with scissors on the right side of the shaved back skin, and a specimen was inserted subcutaneously. Control animals were treated by a sham operation with no implantation. After the operation, the incision was sutured. During the experimental period, body weight and health conditions were monitored.

2.4 Hematological and histological examination

At the end of the experimental period, the animals were anesthetized with diethyl ether, and total blood (approximately 4–5 ml) was collected from the abdominal aorta. The hematological data, such as red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), platelet count (PLT), and white blood cell count (WBC), in the blood sample were measured by a fully automated blood cell counter (M-2000, TOA Medical Electronics Co., Ltd. [Today's Sysmex Co.], Hyogo, Japan). In addition, differentiation of white blood cells was examined using an automated hematology analyzer (MICROX HEG-120A, Omron Tateisi Electronics Co., Tokyo, Japan; Sysmex Co. is presently acquiring Omron's blood image analysis business). According to the common method, spleen, liver, kidney, and the tissue surrounding the specimen were excised from each sacrificed animal. These tissues, along with the specimen, were fixed in 10% formalin and embedded in paraffin. After the specimen was removed gently, the tissue was sectioned and stained with hematoxylin and eosin for microscopic examination. The histological sections were examined microscopically, and the tissue responses—*i.e.*, the distributions of fibroblasts, neutrophils, eosinophils, macrophages, giant cells, lymphocytes, and plasma cells—in the fibrous capsule that formed around the specimen were recorded, as was the infiltration of inflammatory cells around the capsule.[26,29] The intensity of these histological items was ranked as − = no frequency (0), + = low frequency (1), ++ = moderate frequency (2) and +++ = high frequency (3). The total score for the eight items was derived. The thickness of the fibrous capsule that formed around the specimen was also measured by a micrometer.[29] The thickness was determined in the orthogonal direction of the boundary between the capsule and the hole left by the specimen, and expressed as a mean value of 10 spots.[29]

2.5 Evaluation of sensitization response

At 8 months after implantation, a 50 µl aliquot of 2% titanium chloride ($TiCl_4$) in ethanol, 10% zirconium chloride ($ZrCl_4$) in 70% ethanol solution, or 0.02% potassium dichromate ($K_2Cr_2O_7$) in 25% ethanol solution was applied to the shaved skin of the back using a closed-patch dressing (Trii Pharmaceutical Co., Ltd., Tokyo, Japan) for 24 h. Salt of each metal was used as an application chemical for the determination of skin reaction in the patch testing.[26,30] The challenge concentrations of metal salts were determined by a preliminary irritation test, and they were the maximum concentrations to not elicit responses in normal animals. An application vehicle for metal salts at each concentration was prepared to maximize the content of ethanol in water. The skin reactions of erythema and oedema were evaluated by visual assessment at 24 h after removing the closed-patch dressing.

After 8 months of implantation, the spleen was excised and placed on a sterile 200-mesh stainless steel gauge in a 35 mm plastic dish. After 2 ml of Hanks' balanced salt solution (pH 7.4, Sigma-Aldrich Inc., St. Louis, MO, USA) was added to the dish, the spleen was crushed mechanically using

Table 1 Body and organ weights of rats after implantation of each material for 8 months.

Material	Weight (g) (Mean ± SD, n = 6)					
	Body	Thymus	Spleen	Liver	Kidney	
					Right	Left
Absolute weight						
Control	213.8 ± 16.3	0.085 ± 0.009	0.35 ± 0.03	5.15 ± 0.47	0.61 ± 0.04	0.62 ± 0.04
Ti	223.7 ± 20.7	0.086 ± 0.010	0.39 ± 0.01*	5.63 ± 0.59	0.65 ± 0.05	0.65 ± 0.04
Ti–Zr	221.0 ± 12.4	0.082 ± 0.013	0.37 ± 0.01	5.78 ± 0.32*	0.64 ± 0.03	0.64 ± 0.03
Zr	231.5 ± 8.1*	0.091 ± 0.013	0.38 ± 0.01*	5.30 ± 0.39	0.65 ± 0.05	0.64 ± 0.05
Cr	227.7 ± 7.4	0.087 ± 0.012	0.38 ± 0.02	5.96 ± 0.26**	0.67 ± 0.03*	0.65 ± 0.04
Relative weight (organ/body × 1000)						
Control		0.40 ± 0.03	1.66 ± 0.19	24.2 ± 2.5	2.88 ± 0.16	2.90 ± 0.10
Ti		0.39 ± 0.03	1.75 ± 0.13	25.2 ± 1.8	2.93 ± 0.08	2.92 ± 0.20
Ti–Zr		0.37 ± 0.05	1.67 ± 0.10	26.2 ± 0.7	2.88 ± 0.10	2.92 ± 0.10
Zr		0.39 ± 0.05	1.66 ± 0.10	22.9 ± 1.1	2.82 ± 0.15	2.77 ± 0.20
Cr		0.38 ± 0.05	1.69 ± 0.12	26.2 ± 1.2	2.94 ± 0.09	2.87 ± 0.12

*, **: Significantly different from the control group ($p < 0.05$, $p < 0.01$).

Table 2 Hematological data of rats implanted with alloys.

Item	Group				
	Control	Ti	Ti–Zr	Zr	Cr
RBC ($\times 10^4$/ml)	812 ± 27	729 ± 13*	765 ± 24*	779 ± 37	769 ± 16*
HGB (g/dl)	14.8 ± 0.5	13.6 ± 0.2*	14.4 ± 0.4	14.6 ± 0.6	14.4 ± 0.2
HCT (%)	41.8 ± 1.1	38.2 ± 0.8*	40.0 ± 1.7	40.3 ± 1.8	39.9 ± 0.8*
MCV (fl)	51.5 ± 0.6	52.4 ± 0.4*	52.3 ± 0.8	51.7 ± 0.3	51.9 ± 0.2
MCH (pg)	18.2 ± 0.2	18.7 ± 0.3*	18.8 ± 0.2*	18.8 ± 0.2*	18.8 ± 0.3*
MCHC (g/dl)	35.4 ± 0.5	35.6 ± 0.7	35.9 ± 0.5	36.4 ± 0.2*	36.1 ± 0.5*
PLT ($\times 10^4$/µl)	61.1 ± 4.9	65.8 ± 5.7	66.3 ± 6.8	70.8 ± 3.1*	60.0 ± 4.2
WBC ($\times 10^2$/µl)	20.0 ± 7.0	14.0 ± 2.0	18.0 ± 4.0	19.0 ± 6.0	15.0 ± 3.0
Differential cell counts (%)					
Neutrophil-band	0.5 ± 0.7	0.1 ± 0.3	0.3 ± 0.5	0.1 ± 0.2	0.1 ± 0
Neutrophil-segmented	31.5 ± 5.8	34.4 ± 7.7	28.8 ± 6.6	28.9 ± 3.8	30.6 ± 4
Basophil	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Eosinophil	2.5 ± 1.3	2.2 ± 1.3	2.5 ± 1.2	1.3 ± 0.8	1.5 ± 1
Lymphocyte	63.1 ± 6.6	61.3 ± 6.7	65.7 ± 5.9	67.8 ± 3.6	65.7 ± 4
Monocyte	2.4 ± 1.0	2.1 ± 1.3	2.8 ± 1.5	1.9 ± 1.3	2.2 ± 2

Data represent mean values ± SD (n = 6).
*Significantly different from the control at $p < 0.05$.

a syringe, and spleen cells were released. The cell suspension was transferred into a 15 ml tube, and the tube was centrifuged at 1200 rpm for 5 min at 4°C. After the supernatant was removed, the cells were treated with 2 ml of 0.83% ammonium chloride-tris solution (pH 7.65) for 5 min at 37°C for hemolysis. The cells were washed twice by centrifuge at 1200 rpm for 5 min with Hanks' balanced salt solution and resuspended in RPMI-1640 culture medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum. The cell suspensions (5×10^5 cells/200 µl) were seeded into 96-well culture plates (three wells per group) and cultured with 10 µl of 10^{-8} mol/L $K_2Cr_2O_7$, 10^{-5} mol/L $TiCl_4$, 10^{-5} mol/L $ZrCl_4$, or 5 µg/mL Con A and 9.25 KBq [^{3}H]methyl thymidine (^{3}HTdR) for 48 h at 37°C in a humidified atmosphere of 5% CO_2 in air. After incubation, the cells were collected from each well on a glass filter (Type Filter Mat 11731,

SKATRON Instruments AS, Lier, Norway) using an automatic cell harvester (Type 11025, SKATRON Instruments AS). The incorporation of ^{3}HTdR into cells (counts per minute, cpm) was determined by liquid scintillation counting (LSC-5101, Aloka Co. Ltd., Tokyo, Japan).

3. Results

3.1 Body and organ weights

After 8 months of implantation, no significant visual changes such as rust or cracks were found on the surface of any specimen. In all implant groups, no decreases in body and organ weights were found compared with the control group (Table 1). The body weight in the Zr-implant group was higher than that in the control group. The thymus, spleen, liver, and kidney of some animals in the implant groups weighed more than those of control animals. However, the organ weights relative to body weight were not significantly different from group to group.
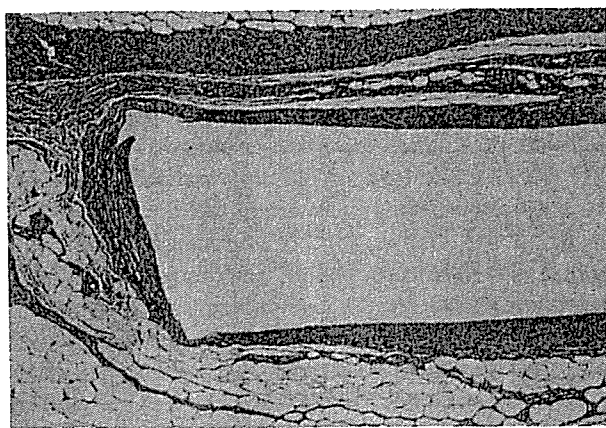
Fig. 1   Histological section of subcutaneous tissue around Ti (hematoxylin and eosin, original magnification × 16). The central lumen was the site of the implant, and the tissue surrounding the lumen formed a fibrous capsule. The thickness of the fibrous capsule was expressed as the mean ± standard deviation (SD) of 10 spots.

3.2   Hematological examination

Table 2 shows the results of analyses of blood constituents, such as RBC, PLT, and WBC counts, and HGB concentration. In the Ti-implant group, slight decreases in WBC count as well as HCT and HGB concentrations were found compared with the control group. Although some implant groups showed small changes in several parameters, none of the specimens showed a clear increasing or decreasing trend in any of these parameters. With regard to hematograms of WBC, no significant difference was found between the control and implant groups.

3.3   Histological examination

Figure 1 is a photograph of a histological section of tissue surrounding a Ti specimen. The central lumen was the specimen's extraction mark. Mature fibroblasts were found to form a distinct capsule between the soft tissue and the Ti specimen. Figure 2 is a typical image of tissues surrounding some specimens at high magnification (×100). The lower part is the specimen's extraction mark. Two of the six animals in the Ti-implant group showed a moderate inflammatory response, and the image of animal no. 3 is shown in (a). Various types of cells infiltrated the fibrous membrane. The photograph in Fig. 3(b) is a tissue image of animal no. 7 of the Ti–Zr alloy-implant group. All animals in this group had levels of tissue reaction similar to those shown in Table 3(b). A fibrous capsule was formed, but the frequency of cell infiltration into the membrane around the Ti–Zr alloy implant was lower than that with the other materials. In the Zr-implant group, the frequency of cell infiltration into the surrounding tissue was higher than that in the Ti–Zr alloy-implant group but lower than that in the Ti-implant group (photograph not shown). The strongest tissue response was observed in the Cr-implant group, and many inflammatory cells were present in the fibrous layer (photograph not shown).

Table 3 summarizes the histological findings of the tissue responses to each material. The distribution frequencies of inflammatory cells or fibroblasts in or around the fibrous capsule were ranked and the total score was derived for each
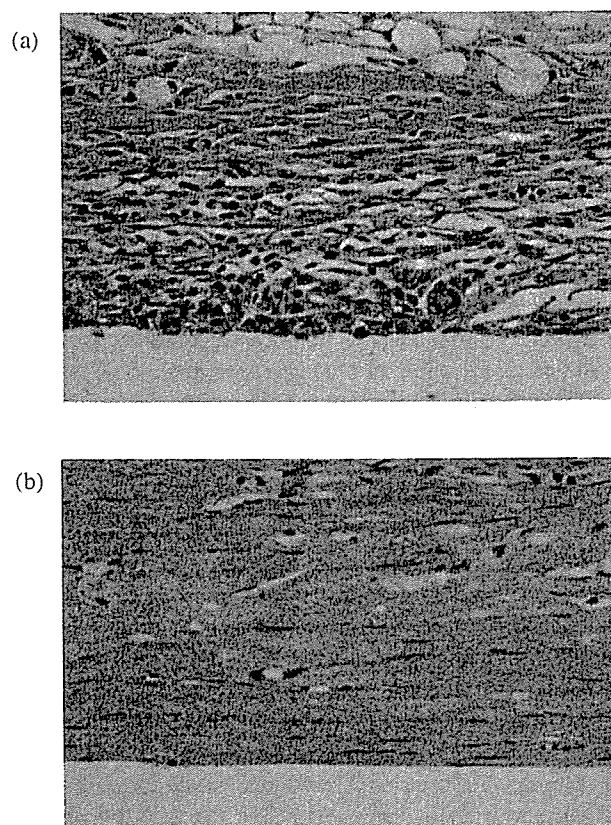

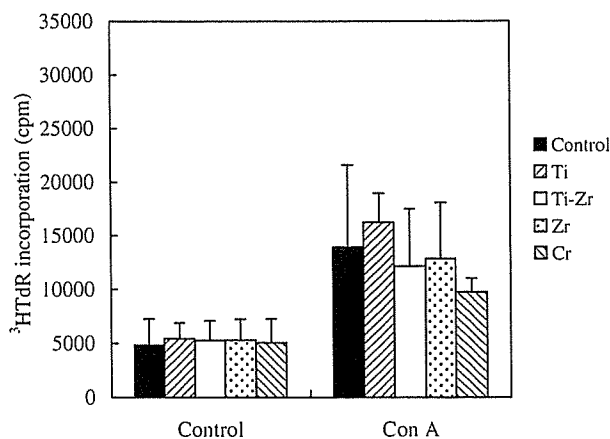Fig. 2   Histological image of tissue around Ti (a) and Ti–Zr alloy (b) at high magnification (×100).

Fig. 3   Con A-induced blastogenesis of spleen lymphocytes in rats implanted with the material for 8 months. Single cell suspension of spleen cells (5×10^5 cells) was cultured with 5 μg/ml Con A and [^{3}H]methyl thymidine (^{3}HTdR) for 48 h. The culture was terminated by automatic cell harvesting, and the ^{3}HTdR incorporation (cpm) was determined. The data are the mean ± SD of 6 animals.

material. The cell distributions in tissues surrounding the materials observed in this study were not as strong as those in our previous implantation study, which used a 4-month experimental period.[26] Large amounts of fibroblasts were observed in all animals in the Cr-implant group and in some animals in the Ti-implant group. Greater numbers of macrophages, the main inflammatory cell types, infiltrated the fibrous capsules around the Cr or Ti specimens compared

Table 3   Histological findings of the tissues around materials implanted for 8 months.

Sample	No.	In fibrous capsule							Around fibrous capsule	Total score	(mean ± SD)	Thickness of fibrous capsule (μm)	(mean ± SD)
		Fibroblasts	Neutrophils	Eosinophils	Macrophages	Giant cells	Lymphocytes	Plasma cells	Inflammatory cells				
Ti	1	+++	+	+	++	+	++	+++	++	15	9 ± 6	83	119 ± 48
	2	+	+	+	+	−	+	−	+	6		71	
	3	+++	++	+	+++	++	+++	++	+++	19		80	
	4	+	+	−	+	−	+	−	+	5		170	
	5	++	+	−	+	−	+	−	+	6		179	
	6	+	−	−	+	−	+	−	+	4		129	
Ti–Zr	7	+	+	−	+	−	+	−	+	5	5 ± 0	119	128 ± 16
	8	+	+	−	+	−	+	−	+	5		121	
	9	+	+	−	+	−	+	−	+	5		140	
	10	+	+	−	+	−	+	−	+	5		108	
	11	+	++	−	+	−	+	−	+	5		153	
	12	+	+	−	+	−	+	−	+	5		126	
Zr	13	+	+	−	+	−	+	−	−	4	5 ± 1	141	153 ± 11
	14	++	+	+	+	−	++	−	+	8		165	
	15	+	+	−	+	−	+	−	+	5		143	
	16	+	+	−	+	−	+	−	+	5		165	
	17	+	+	−	+	−	+	−	+	5		160	
	18	+	+	−	+	−	+	−	−	4		146	
Cr	19	+++	++	+++	++	−	+++	−	++	15	12 ± 6	222	170 ± 36
	20	+++	+	++	++	−	++	−	+	11		161	
	21	++	+	+	+	−	++	−	++	9		167	
	22	+++	+++	++	+++	+	+++	+++	+++	21		198	
	23	++	+	−	+	−	+	−	−	5		118	
	24	+	+	++	+	−	++	−	+	8		155	

Each parameter was scored as − = no frequency (0), + = low frequency (1), ++ = moderate frequency (2), and +++ = high frequency (3).

with the Ti–Zr or Zr specimens. A few giant cells were found in the Ti- and Cr-implant groups, but not in the Zr-implant and Ti–Zr alloy-implant groups. Increased numbers of neutrophils, eosinophils, and lymphocytes were found especially in the Cr-implant group compared with the Ti–Zr alloy- and Zr-implant groups. The total scores in the Ti–Zr alloy- and Zr-implant groups were significantly lower than that in the Cr-implant group.

The capsule membrane formed around the Ti specimen was thinner than those around the Zr and Cr specimens. Individuals varied considerably in membrane thickness in the Ti-implant group, so no statistically significant differences were obtained between the Ti- the Ti–Zr alloy-implant group. The membrane around the Cr specimen was the thickest ($170 \pm 36 \mu m$) among the materials tested and differed significantly from that around the Ti–Zr alloy specimen ($126 \pm 16 \mu m$). A statistical difference in membrane thickness was also found between the Ti–Zr alloy- and the Zr-implant groups.

3.4 Sensitization

A patch test was performed to assess the sensitization to each metal. The animals were topically challenged with 0.02% $K_2Cr_2O_7$, 2% $TiCl_4$, or 10% $ZrCl_4$ solution on the skin. None of the animals exhibited positive skin reactions to any of these metal salt solutions, so sensitization to Cr, Ti, or Zr did not develop as a result of implantation of the metal specimens.

Spleen lymphocyte proliferation is an *in vitro* indicator in the assessment of immune function and provides information useful for diagnosing contact allergy. The proliferation activity without mitogens or metal salts was similar among the test groups. The addition of $K_2Cr_2O_7$ solution did not stimulate lymphocyte proliferation in the Cr-implant group. In the same way, the stimulation index obtained by the addition of $TiCl_4$ or $ZrCl_4$ were almost all 1.0 in the Ti-, Zr-, and Ti–Zr alloy-implant groups (Table 4). Con A-induced blastogenesis was somewhat lower in the Cr-implant group than in the other groups, but no significant difference was observed (Fig. 3).

Table 4 Lymphocyte proliferative responses by stimulation with each metal salt.

Sample	Stimulation index (mean $\pm$ SD)		
	$TiCl_4$ 10^{-6} mol/L	$ZrCl_4$ 10^{-6} mol/L	$K_2Cr_2O_7$ 10^{-9} mol/L
Control	1.04 ± 0.08	1.03 ± 0.06	1.01 ± 0.07
Ti	1.00 ± 0.09	ND	ND
Ti–Zr	0.98 ± 0.03	0.99 ± 0.05	ND
Zr	ND	0.99 ± 0.02	ND
Cr	ND	ND	0.97 ± 0.05

Spleen was collected from each animal, and a single cell suspension of spleen cells was prepared. The cells (5×10^5 cells) were cultured with each concentration of metal salt and 25 μCi [^{3}H]methyl thymidine (^{3}HTdR) for 48 h, and the ^{3}HTdR incorporation (cpm) into cells was determined. A stimulation index, the ratio of ^{3}HTdR incorporation relative to control wells, was derived for each metal salt. The data are mean $\pm$ SD for 6 animals.
ND = not determined.

4. Discussion

In a corrosive environment, component elements of metallic materials are released from the surface as ions and chemical compounds, and some of them may cause local adverse tissue reactions and the development of metal allery.[1,14] Commercially pure Ti has generally good biocompatibility,[14–17] but its mechanical strength is insufficient for use in artificial hip joints.[16,20] Furthermore, the appearance of increased wear debris from Ti has been associated with tissue inflammation.[1,2,31] Elements for Ti alloys are classified into three microstructural categories: α-stabilizers [aluminum (Al), oxygen (O), nitrogen (N), carbon (C)], β-stabilizers [molybdenum (Mo), vanadium (V), iron (Fe), Cr, Ni, Co] and neutral [zirconium (Zr)]. The properties of Ti alloys vary according to the composition of the elements. Ti alloys with α and near-α microstructures exhibit superior corrosion resistance but low ambient temperature strength. The $\alpha + \beta$ and β alloys have high strength and good formability but relatively low corrosion resistance.[18] For example, Ti–6Al–4V alloy was developed as a high-strength material, but its low corrosion resistance in the living body was problematic.[18] Kobayashi *et al.* experimented with Zr, whose chemical properties are similar to those of Ti, and formed an insoluble oxide. They developed a Ti–Zr alloy that has an $\alpha + \beta$ structure and better mechanical properties than commercially pure Ti. Because of its good mechanical properties, such as tensile strength and hardness, Ti–Zr alloy was presented as a new biomedical material for use in artificial joints or bone plates.[20]

Since orthopedic devices are generally implanted into a corrosive environment for anywhere from several months to the lifetime of the patient, long-term evaluation of biocompatibility is necessary. In a previous study, we investigated sensitization of the rat to Cr by implantation of Cr–Fe alloys into a subcutaneous position for 4 months.[26] Lewin *et al.* also evaluated the local response to bone screws in guinea pigs after 4 months' implantation.[32] Oron and Alter examined the corrosion of metal specimens by implantation into rats for 14 months, and found progressive increase in corrosion concomitant with the length of implantation period.[2] In this study, an implantation period of 8 months was used because Ti–Zr alloy and other metal specimens do not easily corrode and release metallic ions. Furthermore, the early inflammatory responses caused by surgical injury could be disregarded.

The results of body or relative organ weights, hematological examination, and mitogen-induced blastogenesis suggest that the implantation of test materials did not cause systemic toxicity or decrease immune activity (Tables 1 and 2, Fig. 3). The membrane thickness around the Cr specimen was significantly higher than that around the Ti–Zr alloy specimen (Table 3). The membranes that formed around the Ti–Zr and Ti specimens were similar in thickness to that around the 316L-type stainless steel (SUS316L) specimen, which is used clinically.[8,26] Fibroblasts migrated to the injury site around the implants in the early phase and increased in density with an increasing amount of extracellular collagen. The tightly formed fibroblast membrane inhibits circulation of biological substances to inside the membrane, possibly

with negative consequences. However, the membrane thickness is related only to fibroblasts but not to other cells, so it has debatable utility as an index of tissue response in implantation studies that cover long periods.

Local tissue response to a material is the most important aspect of the material's biocompatibility, and is mainly related to inflammatory reaction.[15] It can be evaluated by analyzing the cell population or the morphological characteristics of the tissue around the implant.[8,21,22] Ryhänen *et al.* found no qualitative differences in histology between stainless steel, Ti–6Al–4V, and Ti–Ni alloy 26 weeks after implantation in rats.[8] Macrophages are the main inflammatory cell types in a short-term implantation. They play an important role in acute inflammation and probably in determining the final biocompatibility of an implanted material.[33,34] They release various mediators that influence the activities of fibroblasts, lymphocytes, and other cells.[33,34] In addition, macrophages form multinucleated foreign-body giant cells, which constitute evidence of a specific inflammatory response evoked by a foreign substance.[35] Two of the six animals in the Ti-implant group showed a moderate inflammatory response. In all animals in the Ti–Zr alloy-implant group, the frequency of cell infiltration into the membrane was smaller than in other implanted groups (Fig. 2). The numbers of macrophages and inflammatory cells in the fibrous tissue around the Cr and Ti specimens were higher than those around the Ti–Zr and Zr specimens (Table 3). In the Ti- and Cr-implant groups, giant cells were found. These observations indicate that the Cr and Ti specimens induced inflammatory reactions more strongly than did the Ti–Zr and Zr specimens. Neutrophils, eosinophils, and lymphocytes are involved in immune responses.[8,36] The infiltration frequency of these cells into the fibrous membrane was especially high in the Cr-implant group. A small increase in these cells was observed in the Ti-implant group relative to the control group. However, the number and type of infiltrating cells did not differ among the tested materials. The histological examination did not allow us to clearly say whether or not hypersensitive response occurred in an animal. The total scores obtained in the Ti–Zr alloy- and Zr-implant groups were significantly lower than that in the Cr-implant group. The total score in the Ti–Zr alloy-implant group was the same as or less than those of the Ti-implant and SUS316L-implant groups, the latter of which scored 7.3 ± 1.5 in the previous study.[26] These results suggest that the Ti–Zr alloy has good biocompatibility.

The patch test using metal salts is the most widely used method for evaluating metal allergy.[30,37] The *in vitro* lymphocyte proliferation test is also considered useful for assessing allergic conditions or immune activity.[38,39] If a positive reaction appears following the implantation of a metallic device, the contribution of the device to the development of metal sensitivity should be considered. We previously observed that animals became sensitized by the high amount of Cr ion released from the easily corrosive Cr–Fe alloy implanted in them.[5,26] The stable oxide complex layer that formed on the surfaces of Ti, Zr, and their alloys allowed little release of their ions.[16,18,25] Although highly unusual, cases of contact sensitivity to pacemakers which are made of Ti have been reported.[27,28] A positive skin reaction

to titanium chloride was obtained by a sensitization test using guinea pigs.[38] Topical exposure to Ti and Zr salt solutions in rats implanted with Ti, Zr, or Ti–Zr alloy specimens resulted in no skin responses. Ti and Zr salts did not stimulate the proliferation of lymphocytes in these implanted animals (Table 4). None of these materials caused blastogenesis of spleen lymphocytes in the groups implanted with them (Fig. 3). These results indicated that the implantation of the Ti, Zr, and Ti–Zr alloy specimens did not induce sensitization to Ti or Zr ions. This may be explained by the weak sensitization potentials of Zr and Ti ions. Or it may be that the amount of ions released from each specimen was insufficient to cause a sensitization response.

As a result, the Ti–Zr alloy and Zr had better biocompatibility than Ti and Cr. Considering the fragility of Zr, we concluded Ti–Zr alloy as the best material tested in this study. For orthopedic implants, the hardness of metallic materials is important. However, for clinical application, other factors are more important: wear resistance, fretting corrosion resistance, and mechanical/tensile strength such as Young's modulus determine a material's suitability for the targeted position or tissue. In the future, we intend to design new alloys using a Ti–Zr base to serve this purpose.

5. Conclusion

By implanting Ti, Zr, Cr, or Ti–Zr alloy into rats for 8 months, we evaluated the tissue response around the implant and the development of metal sensitization. There was no toxicological change in body or organ weights or in hematological parameters. The tissue inflammatory responses to the Ti–Zr alloy were lower than those to Ti. No sensitization response to the Ti–Zr alloy appeared. The Ti–Zr alloy has better biocompatibility than Ti for use as an artificial surgical implant.

REFERENCES

1) J. J. Jacobs, J. L. Gilbert and R. M. Urban: J. Bone. Jt. Surg. Am. **80** (1998) 268–282.
2) U. Oron and A. Alter: Clin. Orthop. Relat. Res. **185** (1984) 295–300.
3) S. Hierholzer, G. Hierholzer, K. H. Sauer and R. S. Paterson: Arch Orthop. Trauma Surg. **102** (1984) 198–200.
4) U. E. Pazzaglia, C. Minoia, L. Ceciliani and C. Riccardi: Acta Orthop. Scand. **54** (1983) 574–579.
5) T. Tsuchiya, Y. Ikarashi, T. Uchima, H. Doi, A. Nakamura Y. Ohshima, M. Fujimaki, K. Toyoda, E. Kobayashi, T. Yoneyama and H. Hamanaka: Mater. Trans. **43** (2002) 3058–3064.
6) L. Savarino, D. Granchi, G. Ciapetti, E. Cenni, M. Greco, R. Rotini, C. A. Veronesi, N. Baldini and A. Giunti: J. Biomed. Mater. Res. A. **66** (2003) 450–456.
7) A. McNamara and D. F. Williams: Biomaterials. **2** (1981) 33–40.
8) J. Ryhänen, M. Kallioinen, J. Tuukkanen, J. Junila, E. Niemelä, P. Sandvik and W. Serlo: J. Biomed. Mater. Res. **41** (1998) 481–488.
9) M. Uo, F. Watari, A. Yokoyama, H. Matsuno and T. Kawasaki: Biomaterials. **22** (2001) 677–685.
10) L. Kanerva, T. Sipilainen-Malm, T. Estlander, A. Zitting, R. Jolanki and K. Tarvainen: Contact Dermatitis. **31** (1994) 304–307.
11) W. R. Schriver, R. H. Shereff, J. M. Dommitz, E. F. Swintak and S. Civjan: Oral Surg. **42** (1976) 578–581.
12) D. Granchi, G. Ciapetti, L. Savarino, S. Stea, F. Filippini, A. Sudanese, R. Rotini and A. Giunti: Biomaterials. **21** (2000) 2059–2065.
13) L. Kanerva and L. Forstrom: Contact Dermatitis. **44** (2001) 103–104.
14) C. Schmidt, A. A. Ignatius and L. E. Claes: J. Biomed. Mater. Res. **54**

(2001) 209–215.

15) K. Yamaguchi, H. Konishi, S. Hara and Y. Motomura: Spine J. 1 (2001) 204–208.

16) C. E. Wen, Y. Yamada, K. Shimojima, Y. Chino, T. Asahina and M. Mabuchi: J. Mater. Sci. Mater. Med. 13 (2002) 397–401.

17) G. D. Krischak, F. Gebhard, W. Mohr, V. Krivan, A. Ignatius, A. Beck, N. J. Wachter, P. Reuter, M. Arand, L. Kinzl and L. E. Claes: Arch Orthop. Trauma Surg. 124 (2004) 104–113.

18) M. Long and H. J. Rack: Biomaterials. 19 (1998) 1621–1639.

19) J. O. Galante, J. Lemons, M. Spector, P. D. Wilson, Jr. and T. M. Wright: J. Orthop. Res. 9 (1991) 760–775.

20) E. Kobayashi, S. Matsumoto, H. Doi, T. Yoneyama and H. Hamanaka: J. Biomed. Mater. Res. 29 (1995) 943–950.

21) F. Katou, N. Andoh, K. Motegi and H. Nagura: J. Craniomaxillofac Surg. 24 (1996) 155–162.

22) D. F. Williams: J. Bone. Joint Surg. Br. 76 (1994) 348–349.

23) S. H. Lee, F. R. Brennan, J. J. Jacobs, R. M. Urban, D. R. Ragasa and T. T. Glant: J. Orthop. Res. 15 (1997) 40–49.

24) S. D. Rogers, D. W. Howie, S. E. Graves, M. J. Pearcy and D. R. Haynes: J. Bone. Joint Surg. Br. 79 (1997) 311–315.

25) C. Piconi and G. Maccauro: Biomaterials. 20 (1999) 1–25.

26) Y. Ikarashi, T. Tsuchiya, K. Toyoda, E. Kobayashi, H. Doi, T. Yoneyama and H. Hamanaka: Mater. Trans. 43 (2002) 3065–3071.

27) M. A. Peters, A. L. Schroeter, H. M. van Hale and J. C. Broadbent:

28) P. A. Layor, P. A. Revell, A. B. Gray, S. Wright, G. T. Railton and M. A. Freeman: J. Bone. Joint Surg. 73 (1991) 25–28.

29) Y. Ikarashi, K. Toyoda, N. Ohsawa, T. Uchima, T. Tsuchiya, M. Kaniwa, M. Sato, M. Takahashi and A. Nakamura: J. Biomed. Mater. Res. 26 (1992) 339–356.

30) Y. Ikarashi, J. Momma, T. Tsuchiya and A. Nakamura: Biomaterials. 17 (1996) 2103–2108.

31) H. Benghuzzi: Biomed. Sci. Instrum. 32 (1996) 81–86.

32) J. Lewin, J. U. Lindgren and J. E. Wahlberg: J. Orthop. Res. 5 (1987) 604–608.

33) J. M. Anderson and K. M. Miller: Biomaterials. 5 (1984) 5–10.

34) D. G. Vince, J. A. Hunt and D. F. Williams: Biomaterials. 12 (1991) 731–736.

35) A. R. Murch, M. D. Grounds, C. A. Marshall and J. A. Papadimitriou: J. Pathol. 137 (1982) 177–180.

36) M. Thewes, R. Kretschmer, M. Gfesser, J. Rakoski, M. Nerlich, S. Borelli and J. Ring: Arch Orthop. Trauma Surg. 121 (2001) 223–226.

37) P. Thomas, B. Summer, C. A. Sander, B. Przybilla, M. Thomas and T. Naumann: Allergy. 55 (2000) 969–972.

38) S. Torgersen, O. T. Gilhuus-Moe and N. R. Gjerdet: Int. J. Oral Maxillofac Surg. 22 (1993) 246–250.

39) N. J. Hallab, K. Mikecz, C. Vermes, A. Skipor and J. J. Jacobs: Mol. Cell. Biochem. 222 (2001) 127–136.

Contact Dermatitis. 11 (1984) 214–222.

Embryonic Stem Cells Form Articular Cartilage, not Teratomas, in Osteochondral Defects of Rat Joints

Shigeyuki Wakitani,* Hideyuki Aoki,† Yasuji Harada,‡ Masato Sonobe,† Yusuke Morita,‡ Ying Mu,‡ Naohide Tomita,‡ Yukio Nakamura,* Satoshi Takeda,§ Takeshi K. Watanabe,§ and Akira Tanigami¶

*Department of Orthopaedic Surgery, Shinshu University School of Medicine, Matsumoto 390-8621, Japan
†Department of Orthopaedic Surgery, Toho University School of Medicine, Tokyo 143-8540, Japan
‡Graduate School of Engineering, Kyoto University, Kyoto 606-8508, Japan
§Otsuka GEN Research Institute, Otsuka Pharmaceutical Co., Ltd., Tokushima 771-0130, Japan
¶Fujii Memorial Research Institute, Otsuka Pharmaceutical Co., Ltd., Otsu 520-0106, Japan

Embryonic stem (ES) cells are considered to be a potential tool for repairing articular cartilage defects, but so far it has been impossible to cause these cells to differentiate into chondrocytes exclusively, either in vivo or in vitro. To explore a potential new cell source of cell transplantation for articular cartilage defects, we transplanted ES cells into articular cartilage defects in immunosuppressed rats. ES cells (AB2.2 or CCE cells) were transplanted into articular cartilage defects in the patellar groove of immunosuppressed rats treated with cyclosporine. The cells were histologically observed until 8 weeks after transplantation. To determine whether the repair tissue in the defect in the AB2.2-transplanted group was derived from the transplanted cells, the neomycin-resistant gene, which had been transfected into AB2.2 cells but does not exist in rat cells, was used for detection. The cells produced cartilage, resulting in repair of the defects from 4 weeks until 8 weeks after the transplantation without forming any teratomas. The neomycin-resistant gene was detected in every sample, demonstrating that the repair tissue in the AB2.2-transplanted group was derived from the transplanted AB2.2 cells. The environment of osteochondral defects is chondrogenic for ES cells. ES cells may thus be a potential tool for repairing articular cartilage defects.

Key words: Chondrogenic lineage; Hyaline cartilage; Regeneration; Local environment

INTRODUCTION

Articular cartilage covers the surface of moving joints, and its main function is to reduce impact and friction. Because defects of articular cartilage are not completely restored, many attempts have been made to repair articular cartilage defects, but no widely accepted methods have been developed (5).

Embryonic stem (ES) cells (7,17) are one of the most promising tools for tissue repair, because of their unlimited proliferative capacity and ability to differentiate into any tissue or cell (1,2,9,10,13,16). ES cells are thus a potential tool for repairing articular cartilage defects, but so far it has been impossible to cause these cells to differentiate into chondrocytes exclusively, either in vivo or in vitro (12). Because the local environment is important for differentiation into specific tissues or cells (14), we first transplanted ES cells into the joints of immunodeficient mice, but the cells formed teratomas and subsequently destroyed the joints (20). In our next attempt,

reported here, we transplanted ES cells into osteochondral defects in the patellar groove of immunosuppressed rats treated with cyclosporine.

MATERIALS AND METHODS

ES Cell Preparation

AB2.2 prime ES cell kits were purchased from Lexicon Genetics Inc. (Houston, TX). They were cultured according to the manufacturer's instructions on ESQ feeder cells that were supplied with the kit.

CCE ES cells were kindly provided by Dr. Elizabeth J. Robertson and cultured by us on feeder cells (RJ) inactivated by mitomycin C.

Both ES cell lines were obtained from 129/Sv/Ev mice. The ES cells were embedded in a collagen solution (type I collagen obtained from porcine Achilles tendon; Nitta Gelatin, Osaka, Japan) at 4°C at a density of 10^7 cells/ml, and were gelated in 15-µl aliquots at 37°C.

Surgery

Twenty-four rats (F344/DuCrj, Charles River Japan Inc., Yokohama, Japan) weighing about 330–350 g were anesthetized by intraperitoneal injection of ketamin (10 mg/100 g, Sankyo Co., Ltd., Tokyo, Japan) and xylasine (1 mg/100 g, Bayer Co., Ltd., Tokyo, Japan). A hole (2 mm in diameter and 2 mm in depth) was made in the patellar groove of the femur with a hand drill and 15 µl of gel was inserted into the hole. The calculated volume of the defect was 6.28 µl. Because some of the gel protruded from the hole, water was removed from the gel with gauze to make sure it was confined to the hole. Twelve rats received gel with CCE cells transplanted into the right knees and gel without cells into the left knees, while the other 12 rats received gel with AB2.2 cells in the right knees and gel without cells in the left knees.

Immediately after surgery, the recipient animals received daily subcutaneous injections of cyclosporine (14 mg/kg, Novartis Pharma AG, Basel, Switzerland).

We also transplanted the cells embedded in collagen gels into the subcutaneous tissue of severe complex of immunodeficient mice ($n = 2$ for AB2.2 cells and $n = 4$ for CCE cells) and observed them for 8 weeks to determine whether the ES cells formed teratomas.

Histological Evaluation

At 1, 2, 4, and 8 weeks after surgery, three rats from the two groups transplanted with different ES cells were sacrificed each time. The distal femurs were collected, fixed in 10% buffered formalin, and the tissues decalcified and sectioned. Staining was performed with hematoxylin/eosin and safranin-O. The digital images were prepared with Photograb (Fujifilm Co., Tokyo, Japan) and microscopy.

Each sample was graded according to the histological scale described by Wakitani et al. (18). The scale consisted of five categories: cell morphology, matrix staining, surface regularity, thickness of cartilage, and integration of donor with host cartilage. The scores ranged from 0 (normal articular cartilage) to 14 (no cartilaginous tissue). Two-way factorial ANOVA was used for statistical comparative analysis of the overall scores for the four time points between the cell-transplanted groups and the cell-free group. Stat View software (SAS Institute Inc., Cary, NC) was used for analysis, and probability values less than 0.05 were considered significant.

DNA Analysis

DNA was extracted with DEXPAD (TaKaRa, Kyoto, Japan) from a small sample of the repair tissue obtained at each time point. Samples with histologically optimal repair were selected for each postoperative period. We then amplified DNA by means of polymerase chain re-

action to detect the neomycin-resistant gene that had been transfected into AB2.2 cells. The primers for the neomycin-resistant gene were neo p4 (5'-AGGATCTC GTCGTGACCCATG-3') and neo int2 (5'-TCAGAAG AACTCGTCAAGAAGGC-3'), and the size of the reaction product was 250 base pairs. To detect rat cells, we used two other primers: M14103F (5'-GTGGAATGAC GTTCCTTGCT-3') and M14103R (5'-TGGTAACCAA TGTTGAATTGC-3'). The size of the reaction product was 180 base pairs. The primers for the glyceraldehyde 3-phosphate dehydrogenase (GAPD) gene were GAPD-F (5'-AGAAATCCCCTGGAGCTCTATAGGG-3') and GAPD-R (5'-CCGGAATGCCATTCCTGTTAGCTTC-3'), and the size of the reaction product was 250 base pairs.

This study was approved by our Institutional Review Board.

RESULTS

Four weeks after transplantation, osteochondral defects in the patellar groove of the femur were covered by histologically hyaline-like cartilage in three out of three rats transplanted with CCE cells and two out of three transplanted with AB2.2 cells. This result was also observed in two out of three rats with CCE cells and in one out of three rats with AB2.2 cells 8 weeks after the transplantation, whereas rats without ES cell transplantation showed coverage by fibrous tissue or fibro-cartilage (Figs. 1 and 2).

One week after transplantation, ES cells had collected to form numerous small clumps, corresponding to embryoid bodies, in the repair tissue. Two weeks after transplantation, these clumps had disappeared and the cells were distributed more evenly. Cartilaginous tissue was first observed at 4 weeks after transplantation; at this time, thick cartilaginous tissue covered the defect. The cells in the repair cartilage were round and the matrix showed strong metachromasia, indicating that this was hyaline cartilage. Eight weeks after transplantation, the repair tissue still resembled hyaline cartilage, and no cell masses suggestive of teratoma were found in any sample at any time of observation (Fig. 1).

As for the collagen gel without cell implantation (control group), amorphous acellular material, assumed to be the implanted collagen gel, was observed at 1 week. By 2 weeks after implantation, the defects were filled with fibrous tissues, and 4 weeks after collagen gel implantation, metachromasia was observed adjacent to the joint spaces in the fibrous tissue filling the defects, but the cellular morphology and matrix staining indicated that this was fibro-cartilage. Eight weeks after implantation, the repair tissue was still fibro-cartilaginous (Fig. 1).
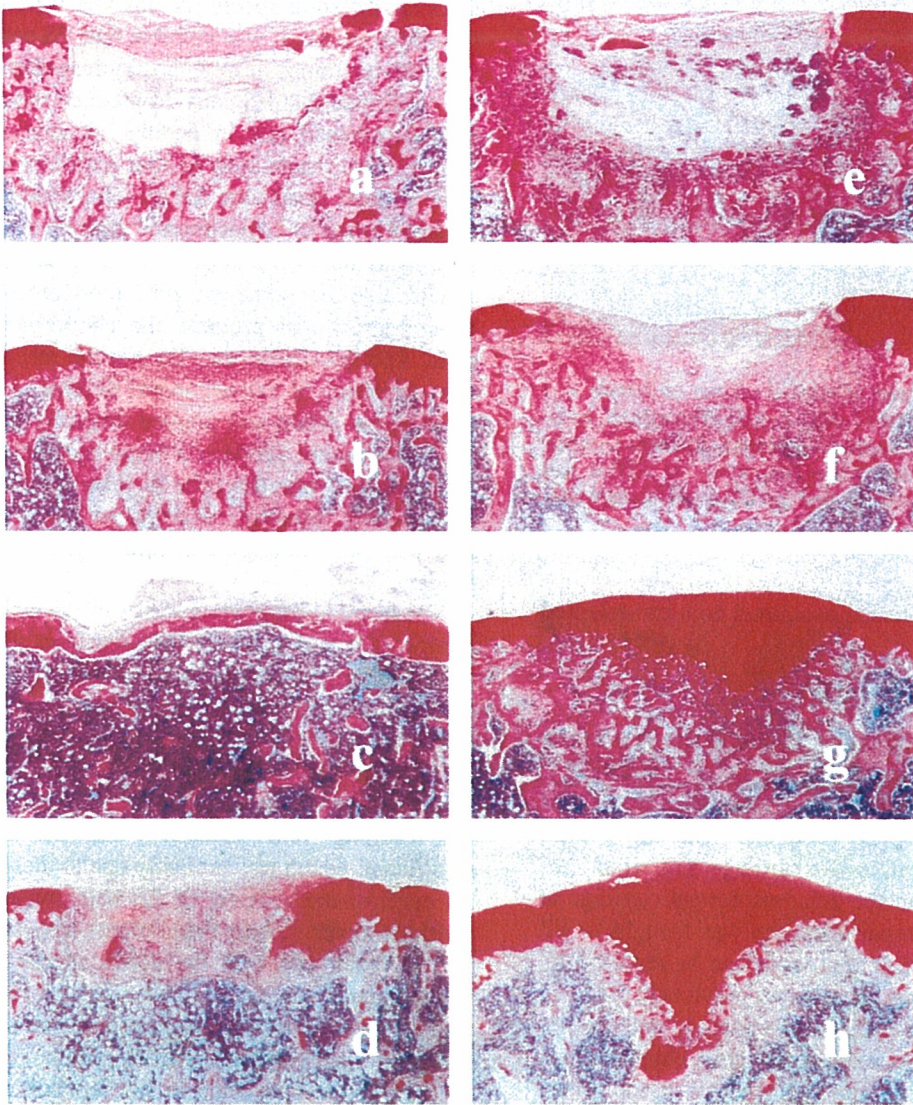
Histological grading scores were determined and used

Figure 1. Photomicrographs of sagittal section of defects at 1 (a, e), 2 (b, f), 4 (c, g), and 8 weeks (d, h) after transplantation of CCE cells (e–h) and cell-free controls (a–d). We selected histologically best repair at each postoperative period. The size of cartilage defects was almost 2 mm in diameter. Safranin-O staining, original magnification ×20.

to compare the repair tissues (Table 1). The differences in the overall scores for the four time points between the cell-transplanted groups and the cell-free group were analyzed with two-way factorial ANOVA. The scores of the CCE cell-transplanted group were significantly better than those of the cell-free control group ($p = 0.0478$), whereas there was no significant difference between those of the AB2.2 cell-transplanted and control groups ($p = 0.0910$).

The two types of cells were also transplanted into the subcutaneous tissue of severe complex of immunodeficient mice. After 8 weeks, we found teratomas in two

out of two mice transplanted with AB2.2 cells and three out of four mice transplanted with CCE cells.

To determine whether the repair tissue in the defect was in fact derived from the transplanted cells, we checked the tissue for the presence of the neomycin-resistant gene, which had been transfected into AB2.2 cells but does not exist in rat cells. The gene was detected in every sample, demonstrating that the repair tissue was derived from the transplanted AB2.2 cells (Fig. 3). We also tried to detect a gene that is present in rats, but not in mice. This gene was detected in one sample, in which the neomycin-resistant gene was also detected,
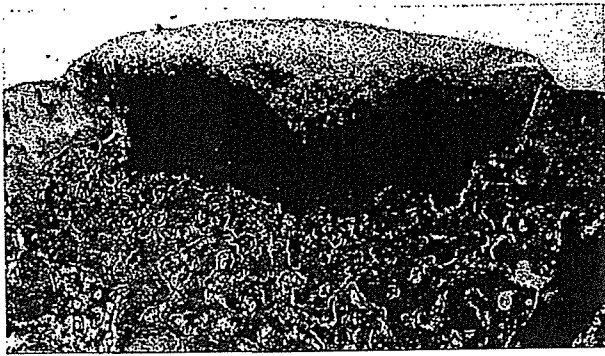
Figure 2. Photomicrograph of sagittal section of defects at 4 weeks after transplantation of AB2.2 cells. The size of cartilage defects was almost 2 mm in diameter. Safranin-O staining, original magnification ×30. The repair by AB2.2 cell transplantation was inferior to that by CCE cell transplantation.

which meant that the tissue contained both transplanted cells and host cells.

DISCUSSION

To the best of our knowledge, this is the first report of articular cartilage repair by ES cells. Interestingly, no sign of tumor growth or nonchondrocyte tissue was observed in the transplant recipients, and the defects were repaired with hyaline-like cartilage. The repair cartilage was thicker than the adjacent normal cartilage at 4 and 8 weeks. The repair cartilage was thought to fill all areas of the defect at first, and then be replaced by the host bone over time, as reported previously (18). ES cells transplanted into the osteochondral defects appeared to differentiate exclusively into chondrocytes, while those transplanted into subcutaneous tissue generated teratomas. Thus, the environment of the osteochondral defect in knee joint was chondrogenic for ES cells.

We have previously reported that AB2.2 cells injected into knee joint spaces of immunodeficient mice formed teratomas (20), whereas in the study presented here they did not generate teratomas in osteochondral defects in rats immunosuppressed with cyclosporine.

Table 1. Histologic Grading Scores for the Repair Tissues

	1 Week	2 Weeks	4 Weeks	8 Weeks
CCE*	14, 14, 14	14, 14, 14	0, 3, 6	1, 6, 14
Control	14, 14, 14	12, 14, 14	12, 12, 14	11, 11, 14
AB2.2	14, 14, 14	14, 14, 14	4, 7, 14	5, 10, 10
Control	14, 14, 14	14, 14, 14	8, 14, 14	13, 13, 14

Scores ranged from 0 = best to 14 = worst.
*Significantly better than the control group.

Because different species of animals were used for the two experiments, it was possible that ES cells would not generate teratomas in immunosuppressed rats. However, we confirmed that ES cells do produce teratomas in immunosuppressed rats in another experiment (data not shown). One reason may be that nutrition was different. Cells in joint spaces are fed by joint fluid not by blood, whereas those in osteochondral defects are fed by both joint fluid and blood in osteochondral defects. It is thus conceivable that some growth factors are released from bone marrow and promote the chondrogenesis of ES cells. Another reason may be that the mechanical stress was different. In osteochondral defects, cells are confined to the defect and pressed down by the patella, so that they cannot produce a large mass that grows out of the defect, whereas cells in joint spaces can expand substantially. Mechanical stress on tissue in the defect may also play an important role in promoting chondrogenesis.

The histological grading scores of the cell-transplanted groups at 4 and 8 weeks differed, and the reason for these differences is thought to be due to technical difficulties in transplantation surgery or differences in immunological reaction of individual rats. We transplanted two kinds of ES cells (AB2.2 and CCE) and both promoted repair of the articular cartilage defect, although that resulting from AB2.2 cell transplantation was not significant, which was probably due to these differences. To confirm the efficacy of ES cell transplantation for cartilage repair, further experiments may be necessary.

The repair tissue in the defect in the AB2.2-transplanted group was proven to be derived from the transplanted cells because the neomycin-resistant gene, which had been transfected into AB2.2 cells but does not exist in rat cells, was detected in the repair tissue. We also detected a gene that is present in rats but not in mice in one of the four samples, in which neomycin-resistant gene was also detected. We may have collected the underlining host tissue in this case or it is possible that the repair tissue consisted of both transplanted cells and host cells. In the other three cases, we concluded that the repair tissue was derived from the transplanted cells, not from the host cells. The transplanted AB2.2 cells differentiated into chondrocytes and formed repair cartilage.

It has been difficult to use ES cells for tissue repair because we could not target the cells exclusively to differentiate into a particular tissue in vitro or in vivo. To obtain homogenous cell populations, it has been possible to induce ES cells with differentiation factors [i.e., neurons and skeletal muscles (4) and glial precursors (6)] or transfect them with vectors that make selective differentiation into cardiomyocytes possible (11). It has also been reported that in vitro differentiation of ES cells into
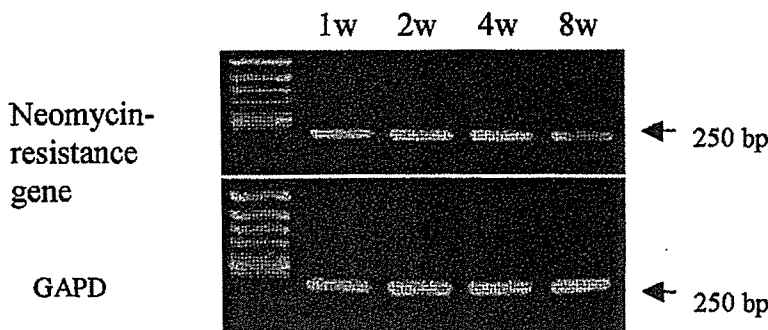
Figure 3. Gel electrophoresis of DNA amplified by the polymerase chain reaction. DNA was extracted from repair tissue at 1, 2, 4, and 8 weeks after transplantation. Neomycin-resistant gene was detected in each sample.

chondrocytes can be promoted by bone morphogenetic protein-2 or -4, but the cell aggregates thus formed contained a variety of cells and not only chondrocytes (12). When ES cells are transplanted into cartilage defects, the formation of cells other than chondrocytes may interfere with the repair process. Although long-term studies will be necessary to comprehensively address the safety of ES cell transplantation, the possibility of using these cells for tissue repair has been expanded by the results of our study. Further optimization of the donor ES cells such as overexpression of factors promoting differentiation may help to meet the challenges involved in clinical application.

We used an immunosuppressive agent in this study because ES cells are xenogeneic. In the case of human application of ES cells, such cells will be allogeneic. Further investigation is required to assess the immune effects of ES cell transplantation on cartilage defects. Various strategies, such as autologous nucleus transplantation into ES cells or HLA-matched ES cell transplantation, may be required to render the clinical use of ES cells feasible.

Recently, autologous cultured chondrocyte transplantation (3,15) and mosaic plasty (autologous osteochondral grafting) (8) have been proven to alleviate symptoms and achieve a certain degree of repair. However, these methods involve the collection of autologous cartilage, which creates new cartilage defects in the peripheral areas. Thus, new cell sources have been investigated, and stem cells, such as mesenchymal stem cells (19) and ES cells, are thought to be among the most promising sources for use in tissue repair because these cells are capable of both proliferation and differentiation.

REFERENCES

1. Ali, N. N.; Edgar, A. J.; Samadikuchaksaraei, A.; Timson, C. M.; Romanska, H. M.; Polak, J. M.; Bishop, A. E. Derivation of type II alveolar epithelial cells from murine embryonic stem cells. Tissue Eng. 8:541–550; 2002.
2. Boheler, K. R.; Czyz, J.; Tweedie, D.; Yang, H. T.; Anisimov, S. V.; Wobus, A. M. Differentiation of pluripotent embryonic stem cells into cardiomyocytes. Circ. Res. 91: 189–201; 2002.
3. 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. 331:889–895; 1994.
4. Brustle, O.; Jones, K. N.; Learish, R. D.; Karram, K.; Choudhary, K.; Wiestler, O. D.; Duncan, I. D.: McKay, R. D. Embryonic stem cell-derived glial precursors: A source of myelinating transplants. Science 285:754–756; 1999.
5. Buckwalter, J. A.; Mankin, H. J. Articular cartilage repair and transplantation. Arthritis Rheum. 41:1331–1342; 1998.
6. Dinsmore, J.; Ratliff, J.; Deacon, T.; Pakzaban, P.; Jacoby, D.; Galpern, W.; Isacson, O. Embryonic stem cells differentiated in vitro as a novel source of cells for transplantation. Cell Transplant. 5:131–143; 1996.
7. Evans, M. J.; Kaufman, M. H. Establishment in culture of pluripotential cells from mouse embryos. Nature 292: 154–156; 1981.
8. Hangody, L.; Kish, G.; Modis, L.; Szerb, I.; Gaspar, L.; Dioszegi, Z.; Kendik, Z. Mosaicplasty for the treatment of osteochondritis dissecans of the talus: Two to seven year results in 36 patients. Foot Ankle Int. 22:552–558; 2001.
9. Jones, E. A.; Tosh, D.; Wilson, D. I.; Lindsay, S.; Forrester, L. M. Hepatic differentiation of murine embryonic stem cells. Exp. Cell Res. 272:15–22; 2002.
10. Kaufman, D. S.; Thomson, J. A. Human ES cells: Haematopoiesis and transplantation strategies. J. Anat. 200:243–248; 2002.
11. Klug, M. G.; Soonpaa, M. H.; Koh, G. Y.; Field, L. J. Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. J. Clin. Invest. 98:216–224; 1996.
12. Kramer, J.; Hegert, C.; Guan, K.; Wobus, A. M.; Muller, P. K.; Rohwedel, J. Embryonic stem cell-derived chondro-

genic differentiation in vitro: Activation by BMP-2 and BMP-4. Mech. Dev. 92:193–205; 2000.

13. Lumelsky, N.; Blondel, O.; Laeng, P.; Velasco, I.; Ravin, R.; McKay, R. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science 292:1389–1394; 2001.

14. McDonald, J. W.; Liu, X. Z.; Qu, Y.; Liu, S.; Mickey, S. K.; Turetsky, D.; Gottlieb, D. I.; Choi, D. W. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. Nat. Med. 5: 1410–1412; 1999.

15. Peterson, L.; Minas, T.; Brittberg, M.; Nilsson, A.; Sjogren-Jansson, E.; Lindahl, A. Two-to 9-year outcome after autologous chondrocyte transplantation of the knee. Clin. Orthop. 374:212–234; 2000.

16. Shiroi, A.; Yoshikawa, M.; Yokota, H.; Fukui, H.; Ishizaka, S.; Tatsumi, K.; Takahashi, Y. Identification of insulin-producing cells derived from embryonic stem cells by zinc-chelating dithizone. Stem Cells 20:284–292; 2002.

17. Thomson, J. A.; Itskovitz-Eldor, J.; Shapiro, S. S.; Waknitz, M. A.; Swiergiel, J. J.; Marshall, V. S.; Jones, J. M. Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147; 1998.

18. Wakitani, S.; Goto, T.; Pineda, S. J.; Young, R. G.; Mansour, J. M.; Caplan, A. I.; Goldberg, V. M. Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. J. Bone Joint Surg. Am. 76:579–592; 1994.

19. 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 10:199–206; 2002.

20. Wakitani, S.; Takaoka, K.; Hattori, T.; Miyazawa, N.; Iwanaga, T.; Takeda, S.; Watanabe, T. K.; Tanigami, A. Embryonic stem cells injected into the mouse knee joint form teratomas and subsequently destroy the joint. Rheumatology 42:162–165; 2003.

Autologous Bone Marrow Stromal Cell Transplantation for Repair of Full-Thickness Articular Cartilage Defects in Human Patellae: Two Case Reports

Shigeyuki Wakitani,* Tomoki Mitsuoka,† Norimasa Nakamura,‡ Yukiyoshi Toritsuka,‡ Yukio Nakamura,* and Shuji Horibe†

*Department of Orthopaedic Surgery, Shinshu University School of Medicine, Matsumoto 390-8621, Japan
†Department of Orthopaedic Surgery, Osaka Rosai Hospital, Sakai 591-8025, Japan
‡Department of Orthopaedic Surgery, Osaka University Medical School, Suita 565-0871, Japan

This study assessed the effectiveness of autologous bone marrow stromal cell transplantation for the repair of full-thickness articular cartilage defects in the patellae of a 26-year-old female and a 44-year-old male. These two patients presented in our clinic because their knee pain prevented them from walking normally. After thorough examination, we concluded that the knee pain was due to the injured articular cartilage and decided to repair the defect with bone marrow stromal cell transplantation. Three weeks before transplantation, bone marrow was aspirated from the iliac crest of each patient. After erythrocytes had been removed by use of dextran, the remaining nucleated cells were placed in culture. When the attached cells reached subconfluence, they were passaged to expand in culture. Adherent cells were subsequently collected, embedded in a collagen gel, transplanted into the articular cartilage defect in the patellae, and covered with autologous periosteum. Six months after transplantation, clinical symptoms (pain and walking ability) had improved significantly and the improvement has remained in effect (5 years and 9 months posttransplantation in one case, and 4 years in the other), and both patients have been satisfied with the outcome. As early as 2 months after transplantation, the defects were covered with tissue that showed slight metachromatic staining. Two years after the first and 1 year after the second transplantation, arthroscopy was performed and the defects were repaired with fibrocartilage. Results indicate autologous bone marrow stromal cell transplantation is an effective approach in promoting the repair of articular cartilage defects.

Key words: Chondro-progenitor cell; Cartilage regeneration; Resurfacing; Fibrocartilage

INTRODUCTION

Articular cartilage defects have a weak potential for self-repair because of the reduced mitotic capacity of chondrocytes in vivo (2). Because some patients with articular cartilage defects may progress to osteoarthritis as described previously, such defects need to be repaired even though their exact natural course remains obscure (6,8,12).

Traditional methods for repair, such as multiple perforations (9), abrasion arthroplasty (3), and microfracture (4), have not produced consistent satisfactory long-term clinical results. Studies using animal models showed that the repair tissue produced with these methods is mainly fibrocartilage, not hyaline cartilage, and is subject to subsequent degradation (5,10,13). Exploration of the feasibility of autologous chondrocyte implantations

has started recently (1), but some problems remain unsolved. These include difficulties in obtaining sufficient chondrocytes, defects associated with autologous cartilage tissue collection, and insufficient histological repair (7). Multiple autologous osteochondral grafting (mosaic plasty) can result in the repair of articular cartilage defects through the formation of hyaline-like cartilage. This procedure is also limited by defects in donor sites, insufficient repair between the grafts, and technical difficulties in resurfacing the original curvature of the joint surface (7,11).

We reported previously that transplantation of autologous bone marrow stromal cells (MSC) expanded in culture could result in the repair of articular cartilage defects in human osteoarthritic knees (16). This method is clinically straightforward to perform because autologous cells can be readily harvested and expanded in culture

without losing their capacity to differentiate into chondrocytes.

The purpose of this study was to evaluate the clinical results obtained with autologous MSC expanded in culture for the treatment of full-thickness chondral defects in human patellae.

CASE 1

The first case involved a 31-year-old female patient. At 23 years of age, the articular cartilage in her left patella had been treated with arthroscopic shaving at another institute because of chondromalacia in her patellae. However, following this surgical procedure, her left knee pain did not diminish and she had to walk with a crutch. She came to our hospital when she was 25 years old because the pain prevented her from walking normally. Physical examination established patello-femoral crepitation and anterior knee pain. The range of motion of the left knee was restricted to between 0° and 130°. The X-ray findings demonstrated narrowing of the patello-femoral joint space and magnetic resonance imaging deficiency of the patellar articular cartilage. After thorough examination of her left knee, we concluded that the other parts of the knee were normal and that the knee pain was due to the injured articular cartilage of the patella. A few months of conservative treatment, such as muscle training, thermotherapy, medication with painkillers, and intra-articular injection of hyaluronic acid, did not result in improvement in the pain. We decided to perform autologous transplantation of MSC expanded in culture to repair the articular cartilage defect in the patella with the informed consent of the patient.

Ten milliliters of heparinized bone marrow was aspirated from the left iliac crest. After erythrocytes had been removed with dextran, the remaining nucleated cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum. After 3 days of culture, attachment of cells was observed and when the medium was changed, nonadherent cells were removed with the medium. After approximately 10 days, the attached cells had achieved subconfluence and were then passaged to expand in culture. After another 10 days, on the day before surgery, 1.8×10^7 cells were collected and embedded (5×10^6 cells/ml) in 0.25% type I acid-soluble collagen from porcine tendon (Nitta Gelatin Inc., Osaka, Japan) and gelated. This gel–cell composite was then cultured overnight in DMEM supplemented with 15% autologous serum.

The transplantation surgery took place on January 27, 1998. After a medial parapatellar approach, all of the fibrous tissue covering the surface of the patella was removed, the subchondral bone was exposed, and multiple perforations using K-wire with a 1.5-mm diameter were made to facilitate bleeding. The area of the full-thickness cartilage defects in the left patella was about 12.0 cm². Because the volume of the collagen with cells was about 3.6 ml, the gel–cell composite was formed with a thickness of 2 mm. The composite was then put in place and covered with autologous periosteum taken from the anterior surface of the tibia with the cambium layer facing the bone marrow. The flap was sutured to the surrounding rim of the normal cartilage or soft tissue with interrupted absorbable sutures. Continuous passive motion was started 3 days after surgery but otherwise the knee was immobilized with a brace for 3 weeks. Partial weight bearing was started 3 weeks and full weight bearing 6 weeks after the operation. The pain in the knee diminished and the patient was able to walk without crutches or a cane 6 months after the operation. The knee range of motion remained somewhat limited from 0° to 120°.

Arthroscopic surgery on March 17, 1998 (7 weeks postoperatively) showed that the patellar articular surface was completely covered with tissue that exhibited slight metachromatic staining upon histological examination.

Arthroscopic surgery on October 25, 2000 (2 years postoperatively) showed that the patellar articular surface was completely covered with cartilage-like tissue, which appeared to have a smooth surface and a firm consistency as established by probing. Histological examination of a tissue specimen taken from the surface of the articular cartilage in the graft area demonstrated the presence of a matrix with strong metachromatic staining but with a fibrous appearance, leading us to conclude that this was fibrocartilaginous repair tissue. At the time of writing this report (5 years and 9 months after transplantation) the patient could walk, run without ambulatory support, do housework without knee pain, and was satisfied with the outcome of the transplantation.

CASE 2

The second case involved a 48-year-old male patient. The articular cartilage of his right patella had been injured in a traffic accident when he was 44 years old, and he came to our hospital 4 months after the injury. He had been suffering from right knee pain that caused him to limp and made stair climbing difficult. Physical examination showed neither patello-femoral maltracking nor ligamentous instability, but retropatellar crepitation accompanied by pain. Computed tomography demonstrated a partial depression in the patella, and arthroscopy showed that the medial area of the patella's articular cartilage was fibrillated.

A careful examination of the knee led to the conclusion that the knee pain was due to the injured articular cartilage of the patella. As for the first case, conservative treatment was initiated but the pain also did not diminish for this second patient. We therefore decided on autolo-

gous transplantation of MSC expanded in culture to repair the articular cartilage defect in the patella with the patient's informed consent.

MSCs (1.4×10^7) were prepared in the same manner as for the Case 1 patient. They were embedded in collagen solution with the same cell density (5×10^6 cells/ml), but in this case they were put on a collagen sheet (Koken Inc., Tokyo, Japan) and gelated. The collagen sheet containing the cells was transplanted. The transplantation surgery was performed on November 12, 1999. The medial part of the articular cartilage was found to be damaged (Fig. 1). The injured articular cartilage was removed, the subchondral bone was exposed, and multiple perforations were made to facilitate bleeding from the bone marrow. The area of the full-thickness cartilage defect in the right patella was about 4.5 cm². Because the sheet measured about 10 cm², almost half of the cells were used. The sheet was put in place and covered with autologous periosteum with the cambium layer facing the bone marrow (Fig. 1). The postoperative physiotherapy program was the same as for the first case except for the omission of immobilization.

Arthroscopic surgery on January 11, 2000 (2 months postoperatively) revealed that the patellar articular surface was completely covered with tissue that was softer than the surrounding normal articular cartilage (Fig. 2).

Six months after the operation, the patient no longer experienced his initial symptoms during daily activities, although the knee range of motion was limited from 0° to 130°.

One year after transplantation, arthroscopy and needle biopsy were performed. Arthroscopy showed that the color and hardness in the graft area were similar to those of the surrounding normal cartilage. Histological examination showed that the repair tissue was fibrocartilaginous (Fig. 3). Fibroblastic cells with spindle-shaped nuclei and scattered chondrocytes with lacunae were also observed. There was no evidence of inflammatory cells or vascular proliferation.

At present (4 years after transplantation), the patient can work without knee pain as a truck driver in the same manner as before the injury and is satisfied with the outcome of the transplantation.

This study was performed in accordance with the ethical standards of the hospital committee on human experimentation and of the World Medical Association Declaration of Helsinki.

DISCUSSION

This article describes autologous MSC expanded in culture transplantation procedures used to treat two patients with large patella articular cartilage defects. Histological examination showed that the defects of the patellar cartilage of both patients were repaired with fibrocartilage and not with complete hyaline cartilage.

However, the repair was much faster than natural repair or repair with conventional marrow stimulation techniques. As early as 2 months after transplantation, the defects were covered with cartilaginous tissue. More importantly, the clinical symptoms were reduced dramatically and remained good for a long time, although both patients continued to show slightly limited knee motion.

It is conceivable that the repair in these cases was to some extent due to multiple perforations or the periosteal flap used simultaneously with the cell transplantation. However, we concluded that cell transplantation was the key contributor to the repair seen in the two cases reported here because of the following reasons. Firstly, it is possible that periosteal transplantation after skeletal maturity may not result in the repair of articular cartilage defects because the chondrogenic potential of periosteum reportedly declines significantly with age (14). Secondly, the clinical outcome has persisted for almost 6 years, although tissues repaired with multiple perforations are reported to result in tissue degradation over time (5,9,10,13).

We previously reported that MSC transplantation for the repair of articular cartilage defects was effective in humans because the arthroscopic and histological repair in the cell-transplanted group was superior to that in the cell-free group (16). In that report, we transplanted MSCs into the articular cartilage defects in the medial femoral condyle of osteoarthritic knees in the same manner used in the cases studies described in this article. Tissue repair was judged to be of higher quality in our precious studies, although the observation period was shorter. Differences in the final location of transplanted materials may also influence the outcome of this approach.

Another procedure for cartilage repair by cell transplantation is autologous chondrocyte implantation, which has been widely performed in the United States and Europe. However, the outcome of repair by means of cell transplantation remains controversial. With autologous chondrocyte implantation, clinical symptoms improved but histological examination showed that repair is insufficient (1,7). Furthermore, it has been reported that with this procedure, repair in the patella was substantially inferior to that in the femoral condyle (1). It is possible that repair by cell transplantation in the patella is inferior to that in the femoral condyle because of certain mechanical properties or the small amount of bone marrow in the patella. Therefore, we concluded that the outcomes of our procedure are not inferior to those of autologous chondrocyte implantation, although the repair in our cases was accomplished with fibrocartilage, not hyaline cartilage.

Moreover, autologous MSC transplantation is clinically much easier to perform because there are no side effects associated with cell collection, which can be per-
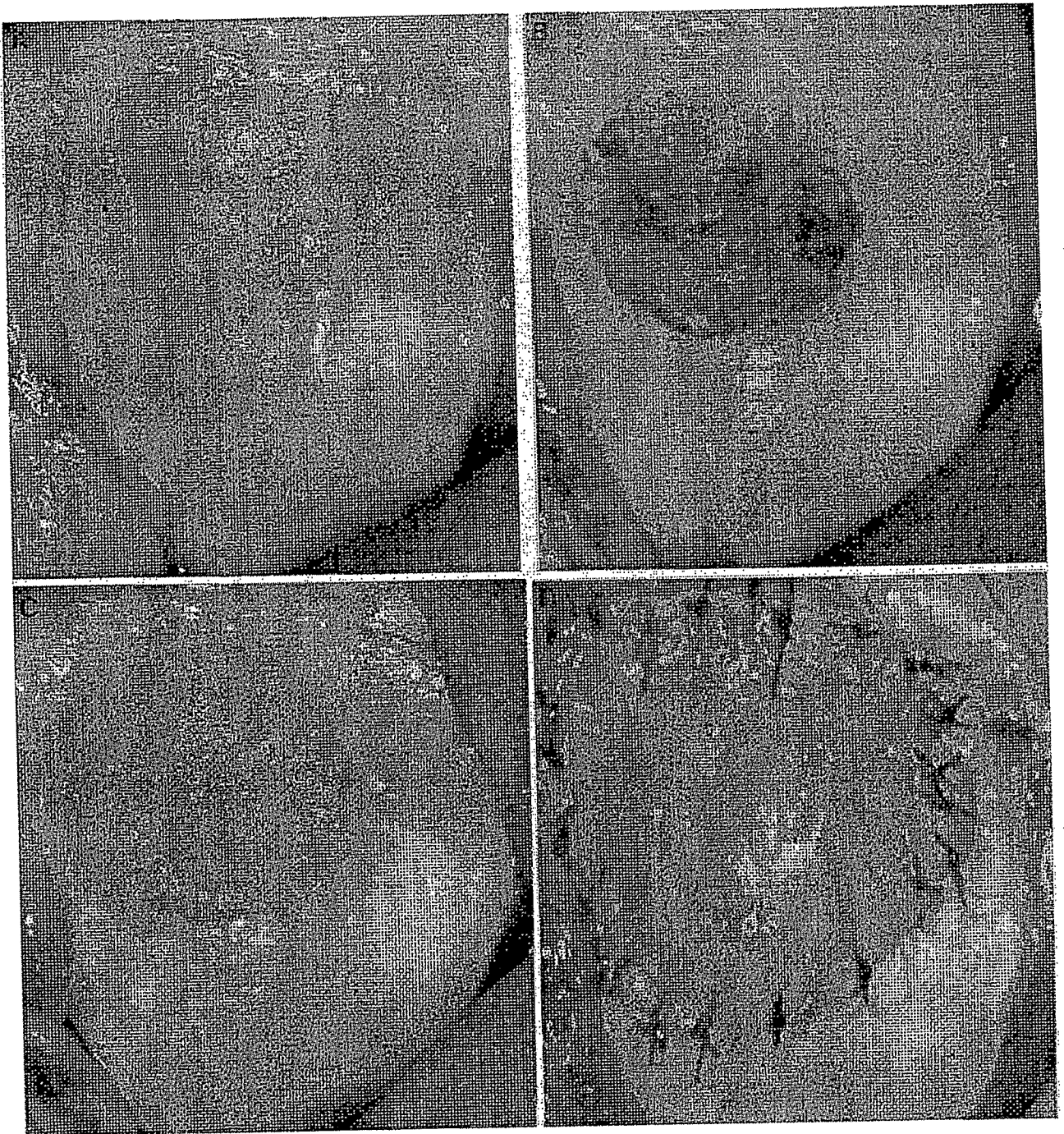
Figure 1. Macroscopic appearances of the patella from the second patient. (A) Damaged articular cartilage. (B) Following removal of damage cartilage. (C) Following implantation of gel–cell composite. (D) Following suturing of autologous periosteum.

formed under partial aesthesia in the outpatient clinic. This also means that surgery is required only once. Furthermore, no bone or cartilage defects remain after collection of autologous cells, and MSCs can proliferate without losing their capacity to differentiate (15).

Cells embedded in collagen gel can be firmly secured in the defect. For the first patient, we used collagen gel only, which was too soft and brittle. For the second patient, we used collagen gel together with a collagen sheet, which was much easier to handle. However, the eventual outcomes of these two patients were very similar.

For these reasons, autologous MSC transplantation can be considered to be a highly promising method for the repair of articular cartilage defects.